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VENOM HEMOLYSIS.*

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GENERAL zoölogical interest as to the structure of snake venoms is augmented by the special importance accredited these secretions in the field of pathology, and a basis for this importance exists in the close structural resemblances which the venoms bear to the toxins produced by pathogenic bacteria. Like the bacterial toxins, the venoms are the highly specialized products of living cells; they are non-crystallizable and chemically undefined; they effect, in minute doses, constant and characteristic pathological changes.

The bacterial toxins possess as a prime characteristic the power of stimulating the production of specific antitoxins. The venoms likewise possess this power, and the phenomena occurring in the process of such an immunization are strictly analogous to those occurring in the immunization with bacterial toxins.

Closely related to antitoxin production is the phenomenon of "toxoid" formation. A bacterial toxin may be so modified by destructive agents that, although deprived of its toxicity, it retains the power of effecting antitoxin production. Venoms similarly treated also display the phenomenon of toxoid formation and herein again strikingly agree with a characteristic property of the true toxins.

Extreme complexity of composition so well recognized for the bacterial toxins is also paralleled in the case of the venoms. While

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in the case of the chemically defined poisons, mercuric chloride for example, the multiplicity of intoxication phenomena is explained by referring them to one and the same poison acting on the several different organs, in the case of the bacterial toxins it must now be recognized that the multiplicity of toxic phenomena depends upon the plurality of dissimilar components which go to make up the composite toxin. Thus, following Ehrlich's conception of special chemical affinities between given cell-groups and individual toxin components, it has been possible to resolve tetanus toxin into at least two toxic components—tetanospasmin and tetanolysin; to which Tizzoni would add as a third constituent a substance responsible for the production of cachexia. In much the same manner and to an equal degree, extreme complexity has been demonstrated as a structural characteristic of the venoms. Thus Myers, employing destructive agents, was able to differentiate a hemolytic from the neurotoxic principle in cobra venom, and more recently there has been described a venom constituent acting specifically on the intimal endothelium of blood vessels.

These, and other marked coincidences between the characteristic properties of the venoms and those of the bacterial toxins are of significant import to workers investigating the bacterial toxins, and especially so since the degree of coincidence seems to justify an expectation that findings as to the structure of venoms may in a degree be applicable to an analysis of the bacterial toxins.

Further, certain limiting conditions which attend a study of the bacterial toxins are less restricting in the examination of venoms. The admixture of culture media, so confusing a complication with the bacterial toxins, is not a factor with the venoms. Then too, the instability of many of the bacterial toxins, so militating against accurate quantitative experiments, although not entirely eliminated in the case of the venoms is reduced to a relatively comfortable working minimum. The venoms offer in addition a certain uniformity of product not approximated by the bacterial toxins.

To a degree influenced by the considerations thus outlined, I have during the past five years conducted investigations as to the structure of venoms, the results of which it is the purpose of this paper to present. These investigations deal primarily with the hemolytic

constituent of venoms and more especially with that of the Indian cobra (*Naja tripudians*).

The experiments were conducted for the most part at the Royal Prussian Institute for Experimental Therapy, and at this point it is a pleasure to express unqualified gratitude for the keen and sympathetic counsel at all times afforded by Professor Ehrlich, the director of that institute. To Dr. Hans Sachs I am indebted for collaboration in certain of the experiments herein referred to and which have been conjointly reported by us in another place. Acknowledgment is also made of generous financial support at various times afforded by the Rockefeller Institute for Medical Research and by the Memorial Institute for Infectious Diseases.

For convenience, the following divisions are observed in the text:

- I. Lytic Action of Venoms upon Serum-free Erythrocytes.
- II. Intracellular Activation.
- III. Lecithin Activation.
- IV. Isolation of Cobra Lecithid.
- V. Serum Activation.
- VI. General Considerations.

I.

LYTIC ACTION OF VENOMS UPON SERUM-FREE ERYTHROCYTES.

The destruction of red blood corpuscles which results in the escape of hemoglobin from the stromata is, for the purpose of this paper, designated as hemolysis, and those components of snake venoms which effect this phenomenon are indicated as venom hemolysins or hemotoxins. In the analysis of the action of these hemolysins, so extensive recourse has been made to quantitative test-tube experiments that a statement of the general methods of procedure in such experiments forms an essential preface to the discussion of the more specific data.

As a criterion by which to grade the hemolytic power of venoms, a constant blood unit was employed as a standard indicator. This unit indicator, representing the amount of blood added to each tube of a series, was arbitrarily fixed as 1 c.c. of a 5 per cent suspension of serum-free erythrocytes in an 0.85 per cent aqueous solution of sodium chloride, prepared as follows:

Freshly defibrinated blood of a normal adult centrifugalized and the supernatant serum (approximately $\frac{1}{2}$ the total volume) withdrawn; the sediment of erythrocytes

suspended in sufficient 0.85 per cent NaCl solution to represent a 1:40 blood dilution (2.5 per cent); this suspension centrifugized and the sediment resuspended in the same amount of salt solution; this lavage repeated at least twice and the resulting sediment finally suspended in sufficient salt solution to represent a 5 per cent suspension relative to the volume of blood as shed.

The venoms and other reagents tested upon this blood unit indicator were also dissolved or suspended in 0.85 per cent NaCl and were added in graduated amounts to tubes in series.¹ The total content of each tube was brought to an approximate volume equivalent of 1 c.c. by the addition, where necessary, of a supplemental amount of 0.85 per cent NaCl solution; then was added the blood indicator (1.0 c.c.), making the total in each tube approximately 2 c.c. Immediately subsequent to the addition of the blood indicator the tubes were thoroughly shaken and placed at 37° C. At this temperature the reaction was allowed to proceed for two hours, after which the tubes were again shaken and placed at 6° to 8° C. for from 14 to 20 hours. The readings of the grade of hemolysis were made at the close of this period and are known to represent the end phase of each reaction.

Coupled with the special conditions indicated for individual experiments, these general lines of procedure give uniform quantitative results. It must be emphasized, however, that with so delicate an indicator as the cytological one here employed, slight deviations from a stated method may be made to produce results in no way comparable.

The hemolytic action of venoms determined by the methods thus outlined, far from being uniform for all bloods, is found to vary markedly with the species of animal from which the blood is obtained. Thus, according to their susceptibility to cobra venom erythrocytes may be divided, primarily, into two groups, viz.: (1) those which are dissolved by cobra venom alone; (2) those which are dissolved by cobra venom only in conjunction with accessory reagents (complements, etc.).

In illustration of these differences the susceptibility and non-susceptibility of the washed corpuscles of several species of the commoner experimental animals and of man to cobra venom are shown in the following table (Table 1).

From this table it is seen that the erythrocytes of the guinea-pig, dog, man, mouse, frog, rabbit, rat, goose, pig, and horse are dissolved by cobra venom alone, whereas those of the ox, sheep, and goat are not dissolved even by relatively large doses of the venom;

¹ The percentage of concentration indicated for the venoms is relative to native venom desiccated *in vacuo* to constant weight.

in fact, no concentration of the venom, however great, effects a solution of the corpuscles of the latter group.

TABLE 1.
SUSCEPTIBILITY OF ERYTHROCYTES OF DIFFERENT SPECIES TO COBRA VENOM.

AMOUNT OF 1 PER CENT COBRA VENOM C.C.	1 C.C. 5 PER CENT SUSPENSION OF ERYTHROCYTES						
	Frog	Dog	Guinea- Pig	Man	Rat	Swine	Mouse
1.0.....	complete	complete	complete	complete	complete	complete
0.1.....	"	"	"	"	"	complete	"
0.05.....	"	"	"	"	"	"	"
0.025.....	"	"	"	"	"	"	"
0.01.....	"	"	"	"	almost complete	marked	medium
0.005.....	"	"	"	"	marked	trace	trace
0.0025.....	"	"	"	almost complete	faint	o	o
0.001.....	"	marked	slight	complete	trace	o	o
0.0005.....	marked	trace	trace	medium	o	o	o
0.00025.....	slight	faint	faint	trace	o	o	o
0.0001.....	o	trace	trace	trace	o	o	o
0.00001.....	o	o	o	o	o	o	o

AMOUNT OF 1 PER CENT COBRA VENOM C.C.	1 C.C. 5 PER CENT SUSPENSION OF ERYTHROCYTES					
	Goose	Rabbit	Horse	Ox	Sheep	Goat
1.0.....	complete	complete	complete	o	o	o
0.1.....	"	complete	complete	o	o	o
0.05.....	"	almost complete	trace	o	o	o
0.025.....	almost complete	slight	faint	o	o	o
0.01.....	marked	o	trace	o	o	o
0.005.....	faint	o	o	o	o	o
0.0025.....	trace	o	o	o	o	o
0.001.....	o	o	o	o	o	o
0.0005.....	o	o	o	o	o	o
0.00025.....	o	o	o	o	o	o
0.0001.....	o	o	o	o	o	o

Table 1 further shows that here, as in general with true toxin hemolysins, there exists between the several susceptible species marked differences in the degree of susceptibility. Thus the amount of venom required to dissolve frog's corpuscles is but $\frac{1}{100}$ the amount required for the solution of rabbit or horse corpuscles. Indeed, as might be expected from the behavior of true toxins, a certain difference in degree of susceptibility is not infrequently found between individuals of the same species. Such differences most frequently occur with the rabbit and horse, and the following table indicates the degree of variation shown by the blood of four rabbits reacting with the same specimen of cobra venom (Table 2).

From this table it is seen that between individuals of the same species there may exist a distinct variation in the lytic dose, a difference which in extreme cases is tenfold in extent. On the other hand, of the susceptible species no individual has been found whose erythrocytes were not dissolved by some concentration of cobra venom, and vice versa, no individual of a non-susceptible species has been found whose erythrocytes could be dissolved by any concentration of cobra venom.¹

TABLE 2.
DIFFERENCES IN SUSCEPTIBILITY TO COBRA VENOM OF ERYTHROCYTES FROM
INDIVIDUALS OF SAME SPECIES.

AMOUNT OF COBRA VENOM (1.0 PER CENT) C.C.	1 C.C. 5 PER CENT RABBIT ERYTHROCYTES			
	Rabbit No. I	Rabbit No. II	Rabbit No. III	Rabbit No. IV
1.0.....	o
0.5.....	faint trace
0.25.....	slight
0.1.....	complete	o	trace	complete
0.075.....	faint trace	complete	"
0.05.....	o	medium	marked	"
0.025.....	o	o	complete	"
0.01.....	o	o	trace	"
0.005.....	o	o	o	marked
0.0025.....	o	o	o	trace
0.001.....	o	o	o	faint trace
0.0005.....	o	o	o	o

Furthermore, of 22 specimens of cobra venom not one was found which failed to dissolve the erythrocytes of all the species designated as susceptible, or which produced hemolysis of any of the corpuscles designated as non-susceptible. In other words, such variations as occur are quantitative and not qualitative.

The underlying structural differences which determine that the erythrocytes of one species shall be dissolved by cobra venom, whereas those of another species shall not, are discussed in other sections of this paper (II and III). At this point it is only indicated that the fact be established that certain species of corpuscles are dissolved by the venom alone, whereas those of other species are not. This fact is fundamental in that the normal susceptibility of any given species of erythrocytes is the basis of all quantitative experimentation as to activation of venoms by complements, etc., and especial

¹ It is intended here to discuss only the blood of normal adults. For differences between fetal and adult blood see H. Sachs, *Centralbl. f. Bact.*, Abt. 1, 1903, 34, p. 686.

emphasis upon this point is indicated in view of contradictions existing in the literature.

Flexner and Noguchi¹ in an early paper concerning the activation of venoms took the position that no erythrocytes are hemolyzed by venom alone. Working with cobra, water moccasin, copperhead, and rattlesnake venoms, and with dog, rabbit, guinea-pig, sheep, ox, and pig erythrocytes, these authors cited as a result "that in no instance were the washed blood corpuscles hemolyzed by venom." Shortly following the appearance of this statement I was forced to conclude from experiments comparable to those given in Table 1 that the erythrocytes of many species, including several of those cited by Flexner and Noguchi, are dissolved by cobra venom alone, and so stated.² Somewhat later, however, in a second paper Flexner and Noguchi³ retained their position that washed erythrocytes in general are not susceptible to hemolysis by venoms alone, and emphasized the possibility of an apparent susceptibility due to insufficient lavage in the preparation of the cells. Such hemolysis by cobra venom as could not be prevented by sufficient washing of the cells, these authors referred to as "partial hemolysis after some hours." Finally, Noguchi⁴ has come to the view that some kinds of corpuscles are dissolved by venom alone.

In view of these contradictions I can but emphasize the fact that cobra venom constantly produces a hemolysis of many species of erythrocytes, which hemolysis cannot rightly be designated as partial and which is not to be referred to a lack of lavage in the preparation of the cells. Since the early work of Stephens⁵ there has been no doubt as to the favorable influence of certain sera upon venom hemolysis and hence as to the necessity of removing the serum before testing the susceptibility of the erythrocytes *per se*. But that a remaining trace of serum is not a factor determining the susceptibility of the erythrocytes tested in the experiments above cited, is evidenced by two facts. The first of these facts is that simple mathematical calcula-

¹ *Jour. Exper. Med.*, 1902, 6, p. 277.

² *Berl. klin. Wchnschr.*, 1902, 39, pp. 886, 918.

³ *Univ. of Penna. Med. Bull.*, 1902, 15, p. 345.

⁴ *Jour. Exper. Med.*, 1907, 9, p. 436.

⁵ *Jour. of Path. and Bact.*, 1900, 6, p. 273. Also thesis published at the University of Cambridge, November, 1898.

tion shows that a single washing of the erythrocytes in the bulk of salt solution indicated so dilutes the admixed serum that the amount of serum contained in 1 c.c. of a 5 per cent suspension is less than the amount necessary to effect activation even by the most potent serum. As a matter of fact two such washings were invariably made. The second fact which eliminates serum activation as a factor in the case of the washed susceptible corpuscles is that, whereas the serum of certain species produces no activation of cobra venom whatsoever, the corpuscles of the species show a distinct susceptibility. It is evident that here the serum, even were it actually present as a result of insufficient washing, could not be held to account for the susceptibility of the erythrocytes. The blood of the rabbit offers such an example and the following table suffices for explicit data (Table 3).

TABLE 3.
EFFECT OF THE PRESENCE OF RABBIT SERUM UPON THE HEMOLYSIS OF RABBIT ERYTHROCYTES BY COBRA VENOM.

AMOUNT OF COBRA VENOM (1 PER CENT) C.C.	1 C.C. 5 PER CENT RABBIT ERYTHROCYTES +		
	Cobra Venom Alone	Cobra Venom + 0.5 c.c. Rabbit Serum	Cobra Venom + 0.05 c.c. Rabbit Serum
0.1.....	complete	o	o
0.075.....	medium	o	o
0.05.....	faint trace	o	o
0.035.....	o	o	o

Here it is seen that whereas the washed erythrocytes of the rabbit are dissolved by venom alone, the presence of serum of the same animal not only does not activate the venom but actually inhibits lysis otherwise occurring.

Finally it may be shown that not only is there a susceptibility of erythrocytes to cobra venom in the absence of serum, but that there exists a susceptibility which is not modified by maximum lavage. The following table shows the susceptibility of the same preparation of rabbit corpuscles at various stages of washing (Table 4).

From this table it may be seen that the susceptibility of these cells is not only independent of serum but also uninfluenced by multiple washings. In view of these facts, the constant results cited in Table 1 stand as a contradiction of the position that washed erythrocytes are not dissolved by venom alone.

It might be argued that the power of cobra venom to effect typical hemolysis of washed erythrocytes is the unusual property of certain anomalous specimens of venom, but in view of the fact that all of the 22 specimens which I have tested are constant in producing this effect I should rather conclude that the normal was here represented and that a specimen which lacked such

TABLE 4.
EFFECT OF REPEATED WASHINGS OF RABBIT ERYTHROCYTES UPON HEMOLYSIS BY COBRA VENOM.

AMOUNT OF COBRA VENOM (1 PER CENT) C.C.	1 C.C. 5 PER CENT RABBIT ERYTHROCYTES				
	Washed 2X	Washed 4X	Washed 6X	Washed 8X	Washed 10X
0.1.....	complete	complete	complete	complete	complete
0.075.....					
0.05.....	marked	marked	marked	marked	marked
0.035.....	medium	medium	medium	medium	medium
0.025.....	slight	slight	slight	slight	slight
0.015.....	trace	trace	trace	trace	trace
0.01.....	o	o	o	o	o
0.01.....					

power represented an anomalous or attenuated venom. Nor should the impression be conveyed that the power of hemolyzing washed corpuscles is peculiar to the venom of the cobra. It is rather a power possessed by venoms in general. With a single exception, the venoms of 10 species of snakes tested show a lytic action for the corpuscles of one or more species of blood. For completeness in illustration of this, the following table is included. The figures parenthetically placed in the last column represent the amount of each venom required for the solution of guinea-pigs' blood (Table 5).

TABLE 5.
ACTION OF DIFFERENT VENOMS UPON SHEEP, RABBIT, HUMAN, AND GUINEA-PIG ERYTHROCYTES.

VENOM OF	BLOOD OF			
	Sheep	Rabbit	Man	Guinea-Pig
<i>Bothrops lanceolatus</i>	o	o	o	o
<i>Trimeresurus anamallensis</i>	o	o	o	+(1.0 mg.)
<i>Crotalus</i>	o	o	o	+(0.5 mg.)
<i>Trimeresurus riukiuanus</i>	o	o	+	+(0.25 mg.)
<i>Ancistrodon piscivorus</i>	o	o	+	+(0.15 mg.)
<i>Bungarus coerulesus</i>	o	o	+	+(0.1 mg.)
<i>Bungarus fasciatus</i>	o	o	+	+(0.1 mg.)
<i>Naja haje</i>	o	o	+	+(0.05 mg.)
<i>Daboia russellii</i>	o	o	+	+(0.035 mg.)
<i>Naja tripudians</i> (Cobra).....	o	+	+	+(0.025 mg.)

From this table it is seen that of the 10 venoms tested, nine dissolve guinea-pig corpuscles, seven dissolve the corpuscles of man, and one dissolves rabbit corpuscles. The corpuscles of the sheep are not dissolved by any venom. It is also to be noted that there exists a certain correspondence between the intensity of the lytic action of a given venom with a susceptible blood, and the number of species of blood which that venom dissolves. Thus cobra venom, whose lytic action for guinea-pig corpuscles is shown to be the greatest, also dissolves the greatest number (3) of bloods tested. Whereas *Bungarus fasciatus*, which requires a dose four times that of cobra venom to dissolve guinea-pig corpuscles, dissolves but two species of blood, and *Trimeresurus anamallensis*, which requires a dose 40 times that of cobra to dissolve guinea-pig corpuscles, dissolves this most susceptible species only. *Bothrops lanceolatus* fails to dissolve any of the species tested. Judged, therefore, both from the extent of their reactions with a given susceptible blood and from the number of species with which they react, cobra venom may be cited as the most intense of those venoms tested, and *Bothrops* venom as the least so.

Summarizing, then, the data as to the action of venoms upon serum-free erythrocytes, it appears that:

1. Venoms vary in hemolytic power according to their species of origin.
2. Venoms in general possess the power of dissolving the erythrocytes of certain species but lack this power for the erythrocytes of certain other species.
3. For cobra venom the susceptible species include guinea-pig, dog, man, mouse, frog, rabbit, rat, goose, pig, and horse, whereas the non-susceptible species include ox, sheep, and goat.

II.

INTRACELLULAR ACTIVATION.

The recognition of two groups of erythrocytes, the susceptible and the non-susceptible, as indicated in the preceding section, suggests an apparent contradiction as to the structure of cobra hemotoxin. The fact that certain erythrocytes are not dissolved by the venom alone, and that these erythrocytes undergo hemolysis upon the addi-

tion of serum, would seem to indicate an amboceptor structure for the hemotoxin. On the other hand, the fact that certain other erythrocytes are dissolved by the venom without the addition of complements, would appear to argue equally well that the hemolysis is a simple toxin complete in itself and not an amboceptor.

In attempting an analysis of this apparent contradiction it might be urged that cobra venom contains two distinct and dissimilar hemotoxins, the one of amboceptor structure acting only in the presence of suitable serum complements, the other a simple toxin requiring no such activation and responsible for the hemolysis of those erythrocytes designated as susceptible. It must then further be assumed that the simple toxin possesses the power of reacting with the corpuscles of certain species only. As a matter of fact, however, the primary assumption of the presence of a simple hemotoxin is not in accord with the experimental data. Thus, as early as 1898 Stephens and Myers¹ showed that whereas certain erythrocytes are dissolved by cobra venom, these same erythrocytes remain undissolved, if the dosage of venom be sufficiently increased. This observation I have been able to extend to washed corpuscles and especially to those of the rabbit (Table 6).

TABLE 6.
NON-LYTIC ACTION OF MAXIMUM DOSES OF COBRA
VENOM WITH WASHED ERYTHROCYTES.

AMOUNT OF COBRA VENOM (1 PER CENT)	1 C.C. 5 PER CENT SUS- PENSION RABBIT ERYTHROCYTES	
	Rabbit A	Rabbit B
1.0.....	o
0.5.....	faint trace
0.25.....	slight
0.1.....	complete	o
0.075.....	faintest trace	complete
0.05.....	o	medium
0.025.....	o	o
0.01.....	o	o

The above table shows that with washed susceptible corpuscles larger doses of venom may fail to effect hemolysis where smaller doses readily do so, and this occurrence contraindicates the assumption of a simple toxin, inasmuch as with simple toxins an increase in dosage implies an increase in toxic action. The phenomenon cited

¹ *Jour. of Path. and Bact.*, 1898, 5, p. 279.

above suggests rather that the hemolysin here acting is of the nature of a complex toxin, the force of the suggestion being in the fact that the single analogous phenomenon thus far observed occurs with a lysin known to be a complement-amboceptor complex. Neisser and Wechsberg¹ showed in the case of bacteriolytic immune sera that with a fixed amount of complement, a sufficient increase in the amount of amboceptor so deviates the complement as to produce a complete inhibition of the bacteriolysis otherwise occurring. In other words, an increase in the amount of amboceptor in the case of a complex bacteriolysin effects an inhibition apparently comparable to the inhibition of hemolysis produced by an increased dosage of venom.

As strikingly, however, as the above-cited experiments may contraindicate the presence of a simple toxin and suggest rather that hemolysis by cobra venom is in all cases effected by a complex hemotoxin, there still remains for explanation the fact that the venom, lacking complements of its own and in the absence of extracellular complements, still effects hemolysis of many species of erythrocytes. Otherwise stated, there remains to be explained the production of hemolysis by an amboceptor in the apparent absence of activating complements, and before final conclusions may be drawn that the lysis of the susceptible corpuscles is due to venom amboceptors, the presence of complements capable of activating such amboceptors must be established.

Cobra venom itself contains no such complements, and in the hemolysis of serum-free erythrocytes, the single remaining hypothetical source of complements is in the erythrocytes themselves. If the susceptible corpuscles do not possess an intracellular complement, the conception of an amboceptor structure for the venom constituent which dissolves such erythrocytes in the absence of complements, must be relinquished. If, on the other hand, susceptible erythrocytes do contain an intracellular complement, the phenomena thus far observed are explainable on the basis of a complex venom hemotoxin: The differences in the susceptibility of erythrocytes may then be referred to differences in their complement content and the failure of maximum doses of venom to hemolyze where smaller doses are effective, may find a possible interpretation in the deviation of the intracellular complement by an excess of amboceptors.

¹ *Munch. med. Wchnschr.*, 1901, 48, p. 697.

Fortunately the correctness of the assumption as to an intracellular complement is subject to a direct experimental test. Should the susceptible corpuscles contain a complement capable of activating venom, or, in other words, of forming a complete hemolysin, the addition of these complement-containing corpuscles to venom and non-susceptible corpuscles might be expected to activate the venom amboceptors not only for the susceptible but for the non-susceptible corpuscles as well. As I have shown elsewhere¹ this is exactly the result which occurs. Thus when ox corpuscles are added to a cobra-venom solution, they remain undissolved; but if there also be added guinea-pig corpuscles, not only are the susceptible guinea-pig corpuscles dissolved, but also the non-susceptible ox corpuscles. Otherwise stated, the addition of the susceptible corpuscles includes the addition of a non-lytic activating substance which in conjunction with the venom effects hemolysis of the non-susceptible corpuscles. For this intracellular activating substance I have employed the term endocomplement. That the activation is effected by an intracellular substance rather than by a physiological action of the cell *per se* is guaranteed by the fact that destruction of the integrity of the cell, as by laking, results in no diminution of the activating power. The following table indicates the activation produced by the endocomplement of laked guinea-pig corpuscles when complementing cobra hemotoxin for the otherwise non-susceptible ox corpuscles (Table 7).

TABLE 7.
ENDOCOMPLEMENT ACTIVATION.

AMOUNT OF ENDOCOMPLEMENT ($\frac{1}{20}$)*	1 C.C. 5 PER CENT SUSPENSION OX ERYTHROCYTES	
	<i>a</i> +0.02 c.c. 1 per cent Cobra Venom	<i>b</i> Without Cobra Venom
1.0.....	complete	o
0.75.....	"	o
0.5.....	"	o
0.25.....	trace	o
0.1.....	o	o

* The endocomplement preparation employed in the quantitative experiments was as a rule obtained as follows: A given amount of full blood was freed of serum by centrifugalization and washing with 0.85 per cent salt solution. The sediment of corpuscles, after washing, was taken up in a given amount of distilled water, either to the original volume of the blood as shed or to a dilution of this volume, designated as $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{10}$, $\frac{1}{20}$, etc., endocomplement. This solution of laked blood then received sufficient NaCl to establish an 0.85 per cent content of the same.

¹ *Berl. klin. Wchnschr.*, 1902, 39, pp. 886, 918.

The activating action of the endocomplement demonstrated for guinea-pig corpuscles in the above experiment is likewise present in the case of all the species of susceptible corpuscles. Moreover, the degree of the activating power of the corpuscles of the several susceptible species varies directly as the degree of susceptibility of these corpuscles to cobra venom alone. Thus among the corpuscles which show the greatest activating power are the highly susceptible corpuscles of man and of the guinea-pig, whereas the much less susceptible corpuscles of the rabbit and the horse possess a relatively slight activating action. On the other hand, in contrast to the susceptible corpuscles, the non-susceptible corpuscles as such possess no activating power. In a few exceptional instances the laking of non-susceptible cells liberates an endocomplement not operative in the intact cell; but in general, non-susceptible corpuscles even when laked possess no activating power. The susceptibility of erythrocytes to cobra venom may be seen, therefore, to be determined by the presence or absence within the cell of an activating substance available for reaction with the venom hemotoxin. Where such an endocomplement is available, as in the case of the guinea-pig, the corpuscles are susceptible; where no such endocomplement is available, as in the case of the sheep, the corpuscles are non-susceptible.

The recognition of complement activation as an essential factor in the hemolysis of the susceptible corpuscles makes apparent the fact that the fundamental process involved both in hemolysis of the susceptible and of the non-susceptible corpuscles is the same, namely, the elaboration of a complete hemolysin from a venom constituent and an activating substance, be it intra- or extracellular. There exists no contradiction of the assumption that it is one and the same venom constituent which, when activated, effects hemolysis both of the susceptible and the non-susceptible corpuscles. The following table summarizes a series of combinations which illustrate the activating action of the endocomplement of several species of susceptible corpuscles (Table 8).

Close upon the discovery of the endocomplement attempts were made to determine its more characteristic properties, among others its thermal reactions. It was found that by heating the laked susceptible corpuscles for one-half an hour at 62° C. the entire activating

action of the endocomplement is destroyed. At 60° C. the inactivation is almost complete (Table 9).

This finding, in itself correct, led to the deduction that the endocomplement, not unlike the general class of serum complements, is

TABLE 8.
SUMMARY OF ENDOCOMPLEMENT ACTIVATION OF COBRA VENOM FOR NON-SUSCEPTIBLE CORPUSCLES.

ENDOCOMPLEMENT FROM	ERYTHROCYTES OF		
	Ox	Goat	Sheep
Rabbit.....	+	+	+
Man.....	+	+	+
Dog.....	+	+	+
Guinea-pig.....	+	+	+
Goat.....	—*	—	—
Ox.....	+	—	—
Sheep.....	—*	—	—

* In a single instance activation was observed with each of these combinations. The intact corpuscles of goat, ox, and sheep, however, lack all activating action.

thermolabile—a deduction, however, which further experimentation showed to be incorrect, and for the reasons here given. The endocomplement of susceptible corpuscles is not dissolved in the aqueous menstruum of a laked blood preparation, but remains bound to the

TABLE 9.
INACTIVATION OF ENDOCOMPLEMENT IN LAKED BLOOD.

GUINEA-PIG ENDOCOMPLEMENT (16)	1 C.C. 5 PER CENT SUSPENSION OF GOAT ERYTHROCYTES + 0.02 C.C. 1 PER CENT COBRA VENOM		
	a) Normal Unheated	Heated ½ Hour at	
		b) 60° C.	c) 62° C.
C.C.			
1.0.....	complete	trace	o
0.5.....	medium	trace	o
0.25.....	slight	o	o
0.1.....	faint trace	o	o

suspended stromata of the corpuscles. This fact is determined with especial ease in the case of guinea-pig blood where the stromata are readily isolated by centrifugalization subsequent to the addition of salt, and the following table (Table 10) gives in detail the relative endocomplement content of the menstruum and of the stromata in comparison with that of the laked blood from which both were derived.

From this experiment it is seen that when the stromata are removed from a preparation of laked susceptible corpuscles the remaining

menstruum is devoid of activating action and that the isolated stromata, on the other hand, possess quantitatively the activating power of the original preparation. Furthermore, attempts to destroy this activating action of the isolated stromata by heat revealed the fact that in the absence of the hemoglobin-containing menstruum, no inactivation

TABLE 10.
ACTIVATING ACTION OF STROMATA.

AMOUNT FROM <i>a), b), c)</i> C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.01 C.C. 1 PER CENT COBRA VENOM +		
	<i>a)</i> Laked Guinea-Pig Blood	<i>b)</i> Hemoglobin-containing Menstruum of Same	<i>c)</i> Stromata of Same
1.0.....	complete	o	complete
0.5.....	"	o	"
0.25.....	"	o	"
0.15.....	"	o	"
0.1.....	slight	o	trace
0.05.....	o	o	o

of the endocomplement occurs at 62° C. This result demonstrated that the endocomplement itself, in contrast to the serum complements, is thermostable. In view of this fact, the apparent destruction of the endocomplement at 62° C. in laked blood cannot be referred to a thermolability of the endocomplement. It is due, rather, to the complicating presence of the hemoglobin-containing menstruum, as shown by the fact that when stromata are returned to this menstruum and the mixture heated at 62° C., the same complete inactivation of the endocomplement occurs as with freshly laked blood. In illustration of these phenomena the following table suffices (Table 11).

TABLE 11.
HEAT RESISTANCE OF ENDOCOMPLEMENT OF ISOLATED STROMATA.

AMOUNT FROM <i>a), b), c)</i> C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.01 C.C. 1 PER CENT COBRA VENOM +		
	<i>a</i> Suspension of Guinea-Pig Stromata Unheated	<i>b</i> Suspension of Stromata Heated $\frac{1}{2}$ Hour at 62° C.	<i>c</i> Suspension of Stromata + Menstruum, Heated $\frac{1}{2}$ Hour at 62° C.
1.0.....	complete	complete	o
0.5.....	"	"	o
0.25.....	"	"	o
0.15.....	"	almost complete	o
0.1.....	trace	trace	o
0.05.....	o	o	o

Supplementary to the above experiment it was also determined that when pure hemoglobin is added to the suspension of stromata, the same inactivation of the endocomplement occurs at 62° C. as in the case of laked blood, indicating that in the latter instance the hemoglobin of the menstruum is the constituent which favors the inactivation of the endocomplement. As a result of the higher temperature, it appears that the hemoglobin binds the endocomplement, rendering the latter unavailable for reaction with the venom hemotoxin. In the first publication concerning the endocomplement¹ in which I also described the activating action of lecithin for cobra hemotoxin, the inactivation of the endocomplement in laked blood was taken to mean that the endocomplement is thermolabile and therefore not identical with the intracellular lecithin. The recognition, however, of the fact that the endocomplement is not actually thermolabile but thermostable, suggested a reconsideration of the possibility of the endocomplement's being lecithin. Were this actually the case, it must then be expected that lecithin heated to 62° C. in the presence of hemoglobin would lose its activating power as does the endocomplement. As a matter of fact this is the result which actually obtains, and the following experiment shows this loss of activating power suffered by a mixture of crystalline horse hemoglobin² and of egg lecithin when heated at 62° C. (Table 12).

TABLE 12.
LECITHIN-HEMOGLOBIN INACTIVATION.

AMOUNT OF LECITHIN- HEMOGLOBIN SOLUTION*	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHRO- CYTES + 0.01 C.C. 1 PER CENT COBRA VENOM +	
	Hemoglobin-Lecithin Solution	
	a) Unheated	b) Heated $\frac{1}{2}$ Hour at 62° C.
1.0.....	complete	o
0.75.....	"	o
0.5.....	"	o
0.35.....	slight	o
0.25.....	trace	o
0.15.....	o	o

* Five c.c. hemoglobin solution + 5 c.c. 0.0125 per cent lecithin.

Further, a hemoglobin solution previously heated at 62° C. for one-half hour inhibits the activating action of lecithin, if allowed

¹ Kyes, *Berl. klin. Wchnschr.*, 1902, 39, pp. 886, 918.

² This preparation of hemoglobin was kindly furnished by Professor Hübner of Tübingen.

to stand one-half hour at 37° C. with the lecithin prior to the addition of the venom and the erythrocytes.

Additional indications as to the lecithin nature of the endocomplement were also seen in the close correspondence of phenomena exhibited in hemolysis by cobra venom when activated by lecithin and by the endocomplement of laked blood. In both these cases, in contrast to the activation by thermolabile serum complements, the hemolysis occurs at 0° C., the hemolysis is relatively rapid, and lastly, there is marked inhibition of hemolysis by cholesterol.¹ The last point is illustrated by the following experiment (Table 13):

TABLE 13.
CHOLESTERIN INHIBITION OF ENDOCOMPLEMENT AND LECITHIN ACTIVATION.

AMOUNT OF CHOLESTERIN SOLUTION C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.01 C.C. 1 PER CENT COBRA VENOM + SINGLE ACTIVATING DOSE OF		
	a) Guinea-Pig Endocomplement	b) Lecithin	c) Guinea-Pig Serum
0.025.....	o	o	complete
0.01.....	trace	o	"
0.005.....	slight	o	"
0.0025.....	complete	complete	"

The above experiment shows that a given amount of cholesterol which produces no inhibition of the activation by guinea-pig's serum completely inhibits the activating action both of the endocomplement and of lecithin.

All told, the similarities in the action of the endocomplement and lecithin were taken to be highly indicative of an identity between these substances, but more conclusive evidence was sought by direct chemical methods. The extraction of susceptible erythrocytes with alcohol showed the endocomplement to be quantitatively recovered in this solvent, and when so recovered to retain its activating action on boiling. Further determinations of the solubilities of the endocomplement, moreover, showed it to be soluble in ether and chloroform and insoluble in acetone, corresponding in these properties with lecithin. On the basis of these data, therefore, and in view of the highly specific activating action of lecithin the conclusion was reached

¹ The cholesterol solution employed in this and other experiments, unless otherwise indicated, was prepared as follows: 1 c.c. of a hot saturated methyl alcohol solution of cholesterol was added to 9 c.c. of a hot aqueous 0.85 per cent NaCl solution. This gave a homogeneous suspension of cholesterol somewhat less than 1 per cent in concentration.

that the endocomplement is lecithin.¹ More recently I have confirmed this deduction by determining that the intracellular activating substance, when extracted by alcohol and refined by acetone precipitation from chloroform and ether, not only represents the activating power of the laked blood, but, by weight, possesses the activating power of lecithin and exhibits a phosphorus content of approximately 4 per cent.

Thus far it has been argued that the susceptibility of certain erythrocytes to venom hemolysis is due to the presence of an endocomplement and that this endocomplement is lecithin. From this position the assumption might easily be reached that those corpuscles which are not susceptible to venom contain little or no lecithin and hence their non-susceptibility. This is not, however, a fact, and the susceptibility and non-susceptibility of erythrocytes must be referred to other factors than the absolute *amount* of lecithin contained by the cell. The recognition of these factors requires an analysis of the relation of lecithin to other constituents of the cell.

That lecithin is a constant component of erythrocytes has long since been recognized and for many species the amount of lecithin within these cells has been determined by direct chemical analysis. Concerning the exact distribution of lecithin within the erythrocyte, however, and its relation to other constituents of the cell no data have been available. The stroma of the intact red blood corpuscles is, according to Ehrlich,² living protoplasm, and in view of this and of the extensive rôle of lecithin in the metabolism of protoplasm in general, the relation of lecithin to the stroma is of marked importance. The lecithin of the red blood corpuscle appears not to be free within the cell and in this it corresponds with lecithin found in various other sites. The lecithin of egg yolk can be extracted but slightly with ether, whereas the total amount may be recovered by alcohol extraction.³ The lecithin in this case is for the most part coupled with the vitellin of the yolk forming a globulin-like substance soluble

¹ Kyes and Sachs, *Berl. klin. Wchnschr.*, 1903, 40, pp. 21, 57, 82.

² *Charité-Annalen*, 1885, 10, p. 136.

³ Cf. Hoppe-Seyler, *Handbuch der physiologisch- und pathologisch-chemischen Analyse*, VII. Auflage (H. Thierfelder, Berlin, 1903), p. 157.

in salt solution but precipitable on dialyzing.¹ The lecithin is recovered from this combination only by alcohol extraction whereupon the vitellin undergoes changes, losing its solubility in salt solution. In the case of sera and of blood corpuscles, the lecithin is likewise but slightly extracted by ether, but is recovered *in toto* by alcoholic extraction. The same holds also for the isolated stromata of the corpuscles.

On the basis of the intimate relation of the lecithin to the cytoplasm of the stroma occurs the simplest explanation of the differences in the susceptibility of the various species of erythrocytes. In section I it was shown that certain species of corpuscles have a marked activating action for venom, whereas others entirely lack this action. All corpuscles, however, contain approximately the same amount of lecithin as shown both by the activating action of their alcoholic extract and by direct chemical analysis. But in view of the fact that the lecithin in all cases is bound to other substances of the stroma, it appears that the firmness of this binding varies markedly with the species. Thus in goat's erythrocytes the binding is so firm that the affinity of cobra hemotoxin is insufficient to dissociate the complex and these erythrocytes, therefore, neither are susceptible to venom alone nor activate venom for other corpuscles. On the other hand the lecithin of guinea-pig's corpuscles is more loosely bound to the stromata and is therefore available for activation of the venom. Hence these corpuscles do activate venom for other corpuscles and are themselves susceptible to the action of cobra venom alone. Analogous differences in the relation of lecithin to the proteins of blood sera exist and will be discussed in a later chapter. These differences will also be shown to determine the availability of the lecithin of a serum for venom activation.

Abderhalden and Le Count² have suggested in contrast to the above explanation that the non-susceptibility of those corpuscles which are not dissolved by cobra venom alone may be due simply to the presence of an intracellular inhibiting substance (cholesterin) rather than to the particular relation of lecithin to the stroma. This suggestion may be dismissed with the state-

¹ Ebenda, p. 369.

² *Ztschr. f. exper. Path. u. Ther.*, 1905, 2, p. 199.

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ment that the total alcohol-ether extraction of the non-susceptible corpuscles shows that in all cases the amount of cholesterol present is, relative to the amount of lecithin, insufficient to produce an inhibition. Substances resisting such extraction would at all events be considered an integral part of the stroma to which the lecithin is bound. Noguchi,¹ on the other hand, considers that the intra-corpuscular lecithin is in no case available for activation of venom hemotoxin, but that it is the activation of the venom by fats and fatty acids within the susceptible cell which elaborates the complete lysis. This point of view arises from the failure to differentiate between the direct activation of venom by lecithin and an indirect action of the fatty acids, fats, *et cetera*, to be discussed in section VI.

The many factors which may modify the combination of the lecithin with the stroma complex and thus influence the susceptibility of the cell have not been comprehensively determined. In this connection, however, it is to be noted that Sachs² has shown that the corpuscles of fetal ox blood are hemolyzed by cobra venom alone, in contrast to the corpuscles of the adult, and Goebel³ has shown that corpuscles which are non-susceptible to venom in 0.85 per cent NaCl are readily dissolved by the same when suspended in an isotonic sugar solution.

III.

LECITHIN ACTIVATION.

In the foregoing chapter reference has been made to an activating action of lecithin for cobra venom. It now becomes of importance to discuss this action in detail.

Calmette⁴ in 1902 showed that certain sera when heated at 62° C. acquire an activating power for cobra venom, not present in the unheated sera. This indication of a thermostabile serum complement appeared of utmost importance and, extending the findings of Calmette, I was able to determine that all sera possess an activating action when heated at from 65°–100° C., and that the activating action in general is more extensive after heating at the higher temperature.

¹ *Jour. Exper. Med.*, 1907, 9, p. 436.

² *Centralbl. f. Bact.*, 1903, 34, p. 686.

³ *Compt. rend. de la Soc. de Biol.*, 1905, 58, p. 420.

⁴ *Compt. rend. de l'Acad. Sci.*, 134, p. 1446.

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In view of the extreme stability displayed by the activating substance there appeared the possibility of its isolation by the more usual chemical procedures. A given quantity of serum therefore was added to ten volumes of alcohol and the mixture well agitated. The resulting precipitate was removed by centrifugalization and the alcoholic extract evaporated *in vacuo*. The residue thus obtained was taken up in an amount of 0.85 per cent salt solution equal to the original volume of serum and tested with cobra venom for its activating action. This action was found to be marked and comparable to that shown by the heated serum. To further determine the solubilities of the activating product, the salt solution preparation was then shaken with ether, with the result that the activating substance was transferred to the ether. From several sera it was thus determined that the thermostabile activating substance was constantly present and that this substance was soluble in alcohol and in ether.

Ether soluble substances have long been recognized as widely distributed constituents of sera and chief among these cholesterin, lecithin, fats, and fatty acids. After negative results with cholesterin I found lecithin to possess a constant, characteristic activating action for cobra venom.¹

As a solvent for the lecithin the purest methyl alcohol was employed, a determination showing that up to 10 per cent this alcohol produces no destruction of red blood corpuscles. From a 1 per cent lecithin solution in this alcohol, dilutions were made in 0.85 per cent salt solution and the activating power of lecithin quantitatively determined. It was found that from 0.0025 c.c. to 0.0035 c.c. of the 1 per cent alcoholic solution (0.000025 gm. of lecithin) was sufficient to activate a given dose of the venom for the hemolysis of 1 c.c. of a 5 per cent suspension of ox or goat corpuscles. The quantitative results are displayed in the following table (Table 14).

The lecithin first employed was that from egg yolk, prepared by Merck of Darmstadt. This preparation was neutral in reaction, soluble in ethyl alcohol, and precipitable from ether by acetone. The latter procedure (Altmann-Henriquez) was employed to obtain a purified product and this product corresponded in its activating

¹ P. Kyes, "Ueber die Wirkungsweise des Cobragiftes," *Berl. klin. Wchnschr.*, 1902, 39, p. 886.

power with the original preparation. A second preparation of lecithin from Riedel of Berlin gave quantitatively the same activation, as did also "agfa" lecithin. Additional preparations of lecithin were available through the kindness of Dr. Bergell of Berlin, Professor

TABLE 14.
LECITHIN ACTIVATION OF COBRA VENOM.

AMOUNT OF LECITHIN 1 PER CENT	0.002 C.C. 1 PER CENT COBRA VENOM+ 1 C.C. 5 PER CENT SUSPENSION OF	
	Ox Corpuscles	Goat Corpuscles
0.005.....	complete	complete
0.0035.....	"	"
0.0025.....	"	medium
0.0015.....	almost complete	trace
0.001.....	slight	o
0.00075.....	o	o

W. Koch of Chicago, and Professor Schulze of Zurich. The preparation furnished by Professor Koch was derived from sheep brain while that prepared by Professor Schulze was from the seeds of leguminous plants. These various lecithins, regardless of their origin and the mode of their preparation, agreed quantitatively in activating power with one another and with the preparations first mentioned. Cephalin from the sheep brain, which according to Koch¹ is a dioxystearyl-monomethyl-lecithin insoluble in alcohol, showed quantitatively the same activating power as the egg lecithin. Cerebrin and protagon, on the other hand, produced no activation (Table 15).

TABLE 15.
COBRA VENOM+CEPHALIN, CEREBRIN, AND PROTAGON.

AMOUNT FROM a), b), AND c)	1 C.C. 5 PER CENT SUSPENSION OX ERYTHROCYTES+ 0.2 C.C. 0.1 PER CENT COBRA VENOM		
C.C.	Cephalin ^a (1 per cent)	Cerebrin ^b (5 per cent)	Protagon ^c (5 per cent)
0.1.....	complete	o	o
0.05.....	"	o	o
0.025.....	"	o	o
0.01.....	"	o	o
0.005.....	"	o	o
0.0025.....	"	o	o
0.001.....	trace	o	o
0.0005.....	o	o	o
0.00025.....	o	o	o
Control without venom:			
0.1.....	o	o	o

¹ *Ztschr. f. physiol. Chemie*, 1902, 36, p. 134.

It was thus made clear that lecithins in general possess a characteristic power of activating cobra venom for non-susceptible corpuscles. The next step was to determine the effect of lecithin upon the hemolysis of susceptible corpuscles. The results show that in all cases lecithin increases the hemolytic action of cobra venom for susceptible corpuscles or in other words, that a sublytic dose of venom for susceptible corpuscles becomes lytic in the presence of an activating dose of lecithin. The following table is an illustration of this action (Table 16).

TABLE 16.
LECITHIN ACTIVATION OF COBRA VENOM FOR SUSCEPTIBLE CORPUSCLES.

AMOUNT OF COBRA VENOM (1 PER CENT) C.C.	1 C.C. OF 5 PER CENT SUSPENSION OF RABBIT ERYTHROCYTES	
	a) Without Added Lecithin	b) +0.2 C.C. 0.01 per cent Lecithin
0.1.....	complete	complete
0.05.....	faint trace	"
0.025.....	o	"
0.01.....	o	"
0.005.....	o	"
0.0025.....	o	"
0.001.....	o	"
0.0005.....	o	"
0.00025.....	o	"
0.0001.....	o	almost complete
0.00005.....	o	slight
0.000025.....	o	faint trace
0.00001.....	o	o
0.000005.....	o	o

Analogous experiments with other susceptible corpuscles showed that for each species of susceptible corpuscles as with the rabbit, two lytic doses are to be observed, the one, the minimal amount of venom which alone effects complete hemolysis, the other, the minimal amount of venom which produces lysis when activated by lecithin. The latter dose is the only true indicator of the amount of hemotoxin present in a native venom and the susceptibility of the corpuscles indicated by this dose I have designated as the "absolute susceptibility." The amount of difference between these two doses varies with the several species but in all cases the dose necessary for lysis without lecithin is many times that required when lecithin is added. The "absolute susceptibility" of susceptible corpuscles does not differ in extent from that of non-susceptible corpuscles.

The activating action of lecithin for cobra venom thus being determined both in the case of the susceptible and the non-susceptible

corpuscles, the general extent of this activating power was tested by extending the experimentation to other snake venoms. At once it was found that without exception lecithin produces the corresponding activation of all the venoms tested, namely, those of *Bothrops lanceolatus*, *Trimeresurus anamallensis*, *Crotalus adamanteus*, *Trimeresurus riukiuanus*, *Ancistrodon piscivorus*, *Bungarus fasciatus*, *Bungarus coeruleus*, *Naja haje*, and *Daboia russellii*. From among these venoms, the three following suffice to illustrate the general extent of the activating action of lecithin (Table 17).

TABLE 17.
LECITHIN ACTIVATION OF BUNGARUS, DABOIA, AND CROTALUS VENOMS.

AMOUNT OF VENOM 0.1 PER CENT a), b), c) c.c.	1 C.C. OF 5 PER CENT SUSPENSION OF OX ERYTHROCYTES +					
	a) <i>Bungarus fasciatus</i>		b) <i>Daboia</i>		c) <i>Crotalus adamanteus</i>	
	I Without Lecithin	II +0.1 c.c. 0.1 per cent Lecithin	I Without Lecithin	II +0.1 c.c. 0.1 per cent Lecithin	I Without Lecithin	II +0.1 c.c. 0.1 per cent Lecithin
1.0.....	o	complete	o	complete	o	complete
0.5.....	o	"	o	"	o	"
0.25.....	o	"	o	"	o	"
0.1.....	o	"	o	"	o	"
0.05.....	o	"	o	"	o	"
0.025.....	o	"	o	"	o	"
0.01.....	o	"	o	"	o	"
0.005.....	o	"	o	"	o	"
0.0035.....	o	"	o	"	o	"
0.0025.....	o	almost complete	o	marked	o	almost complete
0.001.....	o	marked	o	medium	o	medium
0.0005.....	o	medium	o	slight	o	trace
0.00025.....	o	trace	o	faint trace	o	faint trace
0.0001.....	o	o	o	o	o	o

The preceding table shows that whereas no one of the three venoms tabulated produced hemolysis of ox corpuscles when added alone, all of these venoms effected a most extensive hemolysis in the presence of lecithin. Analogous experiments with the other venoms enumerated above gave comparable results. With the exception of those of *Bothrops lanceolatus* and of *Trimeresurus anamallensis*, both of which showed a lower hemotoxin content, all of the venoms tested displayed a most striking agreement as to the extent of their lytic action. In fact so strict is this agreement that 0.003 mgm. approximates the minimal lytic dose of each of these venoms when activated by lecithin as indicated above.

In view of the number of venoms tested and the constancy of

the results, the generalization was indicated that the hemotoxins of snake venoms are invariably activated by lecithin. It then became of importance to determine whether or not animal toxins, from sources other than the snakes, are activated by lecithin. Calmette's¹ finding that the acute lethal action of scorpion poison is inhibited by antivenin suggested that certain analogies of structure exist between the venoms and scorpion poison and following this suggestion I determined the hemolytic action of scorpion poison, kindly placed at my disposal by Professor Treub. Other than a very slight hemolysis of guinea-pig corpuscles, this toxin showed no lytic action for washed corpuscles. Upon the addition of lecithin, however, the scorpion poison, like the venoms, displayed a marked hemolytic action, dissolving all species of corpuscles indifferently. The following table shows such lecithin activation of scorpion poison for ox corpuscles, and indicates the hemolytic value of this toxin to be about $\frac{1}{20}$ that of cobra venom (Table 18).

TABLE 18.
LECITHIN ACTIVATION OF SCORPION POISON.

AMOUNT OF SCORPION POISON (0.2 PER CENT) C.C.	1 C.C. 5 PER CENT SUSPENSION OF ERYTHROCYTES	
	Without Lecithin	+0.2 C.C. 0.1 per cent Lecithin
1.0.....	0	complete
0.5.....	0	"
0.25.....	0	"
0.1.....	0	"
0.05.....	0	"
0.025.....	0	"
0.01.....	0	medium
0.005.....	0	trace
0.0025.....	0	faint trace
0.001.....	0	0

The activating action of lecithin is not therefore to be considered as restricted to snake venoms. It is rather a property bearing relation to toxins in general, at least to those of animal origin. This fact is further illustrated by the more recent findings of Morgenroth and Carpi² who have shown that the hemotoxin of bee poison is also activated by lecithin and it is also probable that the hemotoxin of the

¹ *Ann. de l'Institut Pasteur*, 1895, 9, p. 225.

² *Berl. klin. Wchnschr.*, 1906, 43, p. 1424.

poison of *Trachinus draco* is activated by lecithin. Briot¹ describes the activation of this fish toxin by heated horse serum in which case the activating substance is in all likelihood the lecithin of that serum. Friedemann² has shown also that the secretion obtained from a pancreatic fistula in the dog, although but slightly hemolytic in itself, is markedly hemolytic in combination with lecithin. This appears as a true lecithin activation; the proof is however less conclusive than in the case of the snake venoms, the scorpion toxin, and the bee poison. At all events sufficient evidence is at hand to show that lecithin as an activator plays an extensive rôle with the animal toxins in general.

The cause of the activating action of lecithin was not to be sought in any lytic power of the lecithin itself for, as shown by the following table, lecithins producing the activation tabulated in the above experiments were devoid of lytic action for washed corpuscles (Table 19).

TABLE 19.
NON-LYTIC ACTION OF LECITHINS.

AMOUNT OF LECITHIN a), b), c), d) (1 PER CENT) C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES			
	a) Lecithin	b) Lecithin	c) Lecithin	d) Cephalin
0.2.....	0.	0	0	0
0.1.....	0	0	0	0
0.05.....	0	0	0	0
0.025.....	0	0	0	0
0.01.....	0	0	0	0

This fact that lecithins may be so isolated as to show no hemolytic action is a point which deserves much emphasis in view of the fact that recent workers³ have employed preparations of lecithin which produce a considerable grade of hemolysis, which hemolysis they wrongly interpret as an essential property of lecithin bearing a relation to its activating power. There are, of course, commercial preparations of lecithin which exhibit a distinct hemolytic action, in fact most such preparations do, presumably either because of admixtures or because of split products of lecithin itself. Possibly also among the varieties of lecithins (lecithans, Koch) there are those which in themselves

¹ *Compt. rend. de la Soc. de Biol.*, 1902, 54, p. 1107.

² *Deutsch. med. Wchnschr.*, 1907, 33, p. 585.

³ Cf. Noguchi, *Jour. Exper. Med.*, 1906, 8, p. 547.

are hemolytic, but notwithstanding this possibility, I find myself inclined to regard marked hemolysis on the part of a lecithin preparation as indicative of an impure product. Be this as it may, the fact illustrated by the above tables remains, namely, that lecithin which shows no lytic action of its own effects the activation of venoms and the degree of this activation is that of lecithins in general regardless of their primary lytic action. The activating action of lecithin therefore, as previously stated, is not to be referred to its lytic action.

On the other hand, in view of the fundamental researches of Ehrlich and Morgenroth concerning the relationship of complement and amboceptor in serum hemolysis, together with the conception of the amboceptor nature of venom hemotoxins, advanced by Flexner and Noguchi, the preferable working hypothesis for an analysis of lecithin activation appeared to be the supposition that the lecithin as a complement reacts chemically with the venom (amboceptor) to form the complete lysin. Not that in all details the hemolysis is strictly comparable to all serum hemolysis, for it must be recognized that the venom hemotoxin is not bound to the corpuscles with the readiness usually seen in the case of serum amboceptors,¹ and further, that lecithin is not the typical serum complement, but that in the main a chemical interaction between lecithin and the incomplete venom hemotoxin possessed of a haptophore group results in the elaboration of a complete hemolysin comparable to the complement-amboceptor complex as conceived by Ehrlich. That such is the nature of the reaction between these two substances seemed the more probable also in view of the known tendency of lecithin to unite with proteins, sugar, *et cetera* (Henriquez and Bing).

Experimental substantiation of the hypothesis of a chemical reaction between the venom and lecithin was not far to seek. Lecithin in physiological salt solution is readily extracted by ether; that is to say, the great bulk of lecithin, but not all. In other words, the distribution of lecithin between the two fluids follows the general law

¹ The binding of venom to red blood corpuscles as first described by Flexner and Noguchi (*Jour. Exper. Med.*, 1902, 6, p. 277) has been severely questioned by Lamb (*Scientific Memoirs by Officers of the Medical and Sanitary Departments of the Government of India*, N. S., 1905, No. 17) who found no evidence of any binding whatsoever. I have not been able to effect a binding comparable to that seen in the case of the amboceptors of serum hemolysins or as described by Flexner and Noguchi. On the other hand it is possible by using a *highly concentrated solution* of venom (cobra) to bind sufficient hemotoxin to corpuscles to effect their solution upon the addition of suitable complements (cf. Kyes, *Berl. klin. Wchnschr.*, 1902, 93, p. 886).

commonly recognized as "loi de partage." The addition, however, of a suitable amount of cobra venom to the lecithin-containing salt solution, was found to interrupt the action of this law, so that ether extraction of the mixture recovered but a slight amount of the lecithin. Each of two equal volumes (10 c.c.) of salt solution, A and B, containing a given amount of lecithin was shaken out with ether. Prior to the shaking out, however, 1 c.c. of a 0.1 per cent cobra venom solution was added to solution B and both A and B were allowed to stand for one-half an hour at 37° C. The ether extract of each was evaporated to dryness and the respective residues taken up in 10 c.c. of salt solution. The extracted aqueous solutions, and the resuspended ether residues from the same, were then tested with cobra venom and non-susceptible ox corpuscles with the results shown in the following table (Table 20).

TABLE 20.
EFFECT OF VENOM ON THE ETHER EXTRACTION OF LECITHIN FROM SALT SOLUTION.

The complete activating dose of the standard lecithin solution with 0.1 c.c. 0.1 per cent cobra venom = 0.005 c.c. (=0.025 c.c. of the solutions A and B prior to extraction).

AMOUNT FROM EACH A AND B	1 C.C. 5 PER CENT SUSPENSION OX CORPUSCLES + 0.1 C.C. 0.1 PER CENT COBRA VENOM			
	Solution A Lecithin Alone		Solution B Lecithin + Cobra Venom	
	I Ether Extract	II Extracted Solution	I Ether Extract	II Extracted Solution
10 c.c.	complete	complete	complete	complete
0.5 c.c.	"	"	medium	"
0.25 c.c.	"	"	o	"
0.1 c.c.	"	o	o	"
0.05 c.c.	"	o	o	"
0.025 c.c.	trace	o	o	"
0.015 c.c.	o	o	o	o

From the above procedure it was seen that the addition of cobra venom to a lecithin solution allowed the ether extraction of but one-twentieth part of the lecithin which was extracted from a similar solution in the absence of the venom. From this it appeared that cobra venom has the power of binding lecithin.

A further point taken to be indicative of a chemical reaction between the venom and lecithin was the difference in the reaction of these substances with blood corpuscles when acting singly and when acting in combination with one another. To offer a greater oppor-

tunity for observing the various stages of the process, conditions were instituted tending to inhibit the rapidity of the reaction. These conditions were a low temperature (0° C.) and dilute solutions. It was found that so slight is the power of the native venom hemotoxin to unite with erythrocytes that under the conditions imposed (two hours' contact in dilute venom at 0° C.) no binding of the venom occurs and lecithin was likewise found not to be bound to the corpuscles. On the other hand, corpuscles added to lecithin + venom at 0° were rapidly dissolved (Table 21).

In view of the fact that neither the venom nor the lecithin reacted singly with the cell, the hemolysis occurring in their combined presence was taken to indicate that these substances even at 0° C. react with one another to form a complete hemolysin possessing a greater affinity for the erythrocytes than that shown by the native venom. That the affinity of the cytophile group of the hemotoxin should be increased by an occupation of another side chain of the molecule, is an easily conceivable chemical result—a result which is seen in the analogous phenomenon of the increase in the affinity of the complementophile group of hemolytic serum amboceptors by the union of the cytophilic group with the cell. In the case of the hemolytic sera an exact parallel also is observed. Ehrlich and Sachs¹ have shown instances in which the occupation of the complementophile group of a serum amboceptor increases the affinity of the haptophore group for the receptor of the cell.

The conception of a chemical reaction between the lecithin and the venom hemotoxin (amboceptor) was found to receive further support also from the quantitative relations existing between these substances. If a reaction occurs between the lecithin and venom as between complement and amboceptor, approximately the same quantitative relations might be expected with lecithin and venom as those observed with the serum lysin constituents. In the case of the serum lysins the experiments of von Dungern,² Gruber,³ and Morgenroth and Sachs,⁴ have shown that with relatively greater amounts of amboceptor smaller doses of complement are sufficient to effect

¹ *Berl. klin. Wchnschr.*, 1902, 39, p. 492.

² *Münch. med. Wchnschr.*, 47, 1900, p. 677.

³ *Wien. klin. Wchnschr.*, 15, 1902, p. 387.

⁴ *Berl. klin. Wchnschr.*, 1902, 39, p. 817.

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TABLE 21.

COMBINED ACTION OF LECITHIN AND VENOM AT 0° C.

Complete lytic dose of 0.1 per cent cobra venom with 0.01 c.c. of lecithin solution=0.005 c.c.

Complete activating dose of lecithin solution with 0.1 c.c. 0.1 per cent cobra venom=0.005 c.c.

A

AMOUNT OF VENOM (0.1 PER CENT) TO WHICH CORPUSCLES WERE SUBJECTED AT 0° C. c.c.	1 C.C. 5 PER CENT OX CORPUSCLES+DECREASING AMOUNTS OF COBRA VENOM FOR 2 HOURS AT 0° C. CORPUSCLES THEN SEPA- RATED FROM SUPERNATANT FLUID AND WASHED. THEN 0.01 C.C. LECITHIN SOLUTION ADDED TO:	
	I Washed Corpuscles	II Supernatant Fluid Containing Fresh Ox Corpuscles
0.1.....	faint trace	complete
0.50.....	o	"
0.025.....	o	"
0.01.....	o	"
0.005.....	o	almost complete
0.0025.....	o	o

B

AMOUNT OF LECITHIN SOLUTION TO WHICH CORPUSCLES WERE SUB- JECTED AT 0° C. c.c.	1 C.C. 5 PER CENT OX CORPUSCLES+DECREASING AMOUNTS OF LECITHIN SOLUTION FOR 2 HOURS AT 0° C. CORPUSCLES THEN SEPARATED FROM SUPERNATANT FLUID AND WASHED. THEN 0.1 C.C. 0.01 PER CENT COBRA VENOM ADDED TO:	
	I Washed Corpuscles	II Supernatant Fluid Containing Fresh Ox Corpuscles
0.075.....	faint trace	complete
0.05.....	o	"
0.025.....	o	"
0.01.....	o	"
0.0075.....	o	"
0.005.....	o	o

C

AMOUNT OF COBRA VENOM (0.1 PER CENT) c.c.	1 C.C. 5 PER CENT OX BLOOD+0.025 C.C. LECITHIN SOLUTION+ DECREASING AMOUNTS OF COBRA VENOM AT 0° C. FOR 2 HOURS:		
	I Hemolysis then Present	II Corpuscles in Tubes Showing No Hemolysis Separated from Supernatant Fluid and Washed	
		a) Washed Corpus- cles+0.01 c.c. Lecithin Solution	b) Supernatant Fluid Fresh Ox Corpuscles
0.1.....	complete
0.05.....	"
0.025.....	"
0.01.....	"
0.005.....	faint trace	o	complete
0.0025.....	o	o	medium
0.001.....	o	o	o
0.....	o	o	o

hemolysis and Neisser and Wechsberg¹ have shown that in the case of certain bacteriolytic sera, an extreme excess of amboceptors may inhibit the action of a minimal amount of complement. Relations analogous to these do exist between venom and lecithin. It was found that with an extreme amount of venom, a greater amount of lecithin is required to effect hemolysis than when less venom is employed. In other words, with a fixed amount of lecithin an increase in the amount of venom beyond a certain point inhibits the lysis. This phenomenon is displayed in the experiment tabulated below (Table 22).

TABLE 22.
QUANTITATIVE RELATIONS BETWEEN COBRA VENOM AND LECITHIN.

AMOUNT OF LECITHIN SOLUTION 0.2 PER CENT C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES +	
	a) 0.4 C.C. 5 per cent Cobra Venom	b) 0.1 C.C. 0.1 per cent Cobra Venom
0.05.....	complete	complete
0.035.....	medium	"
0.025.....	slight	"
0.015.....	faint trace	"
0.01.....	o	"
0.0075.....	o	medium
0.005.....	o	trace
0.0035.....	o	o

In this experiment it is seen that the same amounts of lecithin produced less hemolysis with an extremely large dose of venom than with $\frac{1}{200}$ of that amount. Also that where the larger dose of venom failed to effect hemolysis with a given amount of lecithin, an increase in the amount of lecithin resulted in hemolysis. This phenomenon is analogous to that observed by Neisser and Wechsberg for the bacteriolytic sera which is explained on the basis of a chemical binding of the complement by an excess of amboceptors which do not enter into relation with the cell receptors. Were the action of the venom that of indirectly rendering the corpuscles susceptible to a lytic action of lecithin as such, an increase in the amount of venom would be expected to increase this susceptibility. Such is not the case. Likewise if the lecithin were assumed to act independently on the cell to make it more susceptible to venom, the susceptibility of the cell would not be expected to be less for the larger dose of venom.

¹ *Munch. med. Wchnschr.*, 48, 1901, p. 697.

The results just discussed are those obtained however only when an extreme excess of venom (amboceptor) is employed. With less excessive doses of the venom it was found that the quantitative relations between lecithin and venom are comparable to those given by Morgenroth and Sachs (*loc. cit.*) for complement and amboceptor in the hemolytic sera; namely, that within limits the larger the dose of venom the smaller the amount of lecithin required to effect hemolysis, and vice versa, that the larger the amount of lecithin, the smaller the amount of venom required. The following table (Table 23) indicates these quantitative relations in detail.

TABLE 23.
QUANTITATIVE RELATIONS BETWEEN COBRA VENOM AND LECITHIN.

A. 1 C.C. 5 PER CENT OX CORPUSCLES		B. 1 C.C. 5 PER CENT OX CORPUSCLES	
Amount of Cobra Venom (1 per cent) c.c.	Amount of Lecithin (0.025 per cent) Required for Activation c.c.	Amount of Lecithin (0.025 per cent) c.c.	Amount of Cobra Venom (1 per cent) Required for Complete Hemolysis
0.01.....	0.035	0.3.....	0.00001
0.001.....	0.05	0.06.....	0.0001
0.00025.....	0.075	0.06.....	0.005
0.0001.....	0.1		
0.00001.....	0.5		

From these results it appears that the quantitative relations between lecithin and venom correspond closely with those observed for complement and amboceptor in general and are indicative of a chemical reaction between these substances.

Additional and more complete evidence as to a chemical reaction between lecithin and the venom was found in the fact that when lecithin and venom are mixed and allowed to stand some hours prior to the addition of blood corpuscles, hemolysis occurs much more rapidly upon the addition of corpuscles than when venom, lecithin, and corpuscles are mixed at the same time. When lecithin and corpuscles or venom and corpuscles are similarly mixed, no corresponding elimination of the incubation period results. It appears then that the incubation period represents the time required for a reaction between lecithin and venom and that when a period is afforded for this reaction previous to the addition of blood, the complete toxin is formed (complement-amboceptor complex) so that

upon the addition of the corpuscles the hemolytic action is relatively rapid.

From the total data here indicated the deduction was made that the venom and lecithin react chemically to form a complete hemotoxin and this hemotoxin was designated as "cobra lecithid." The ultimate proof, however, of such a process was recognized to be in the actual isolation of the complete hemotoxin and this proof is advanced in the following section dealing with the production and isolation of cobra lecithid.

IV.

ISOLATION OF COBRA LECITHID.

Attempts to analyze the phenomenon of activation as displayed in serum lysins have been rendered the more difficult by the inadaptability of the lytic substances to direct chemical analysis. The most ingenious of biological experiments have been elaborated to substantiate this or that hypothesis as to the exact mechanism of serum activation, but even where best controlled, the results are to be considered tentative, pending a more satisfactory chemical determination of the reagents participating.

The recognition of the activating action of lecithin for venom hemotoxin afforded the first opportunity of analyzing an activation in which a reagent of known chemical structure was involved. Moreover, the relative stability both of the venom hemotoxin and of the lecithin offered an opportunity for chemical procedure far more favorable than that presented by the serum lysins. Convinced by the biological experiments cited in the previous section that the activation of the venom hemotoxin by lecithin was essentially a chemical reaction between these substances, I therefore attempted the isolation of the assumed end product of this reaction, cobra lecithid, by direct chemical methods.

In the absence of a common solvent for venom and lecithin a reaction between these substances was invited by emulsifying an aqueous solution of venom with a chloroform solution of lecithin. In detail the method as first employed was as follows:¹

Forty c.c. of a one per cent solution of cobra venom in 0.85 per cent salt solution and 20 c.c. of a 20 per cent solution of lecithin in chloroform were brought together

¹ Cf. Kyes, *Berl. klin. Wchnschr.*, 1903, 42, p. 21.

in a container of about 100 c.c. capacity. This mixture of chloroform lecithin solution and aqueous venom solution was vigorously agitated in a shaking apparatus for two hours. Subsequently to this the resulting emulsion was centrifugalized for from $\frac{3}{4}$ to 1 hour in an electrical centrifuge with an arm length of five inches and showing 3,000 revolutions to the minute. From this centrifugalization there resulted a distinct separation of the aqueous solution from the chloroform solution, the line of separation being sharply marked by a compact, clearly defined intermediate emulsion zone less than 1 mm. in thickness. The supernatant aqueous portion was removed from the underlying chloroform by means of a fine pipette. The chloroform portion was likewise regained

TABLE 24.

CONVERSION OF NATIVE HEMOTOXIN INTO COBRA LECITHID.

1 c.c. 5 per cent suspension ox erythrocytes + 0.2 c.c. 0.1 per cent lecithin.

Amount in c.c.	A Native Cobra Venom (Control) 0.001 per cent	B Same Venom Shaken Once with Chloroform Lecithin Solution 0.1 per cent	C Same Venom Shaken Twice with Chloroform Lecithin Solution 1.0 per cent	D Cobra Lecithid Precipitated by Ether from Chloroform Leci- thin Solution 0.002 per cent*
1.0.....	complete	complete	o	complete
0.75.....	"	"	o	"
0.5.....	"	"	o	"
0.35.....	"	"	o	"
0.25.....	"	"	o	"
0.15.....	"	"	o	"
0.1.....	almost complete	"	o	"
0.075.....	marked	"	almost complete
0.05.....	slight	"	marked
0.035.....	trace	almost complete	slight
0.025.....	almost o	medium	trace
0.015.....	o	slight	almost o
0.01.....	o	trace	o
0.0075.....	o	almost o	o
0.005.....	o	o	o
0.0035.....	o	o	o
0.0025.....	o	o	o
0.0015.....	o	o	o
No. of lytic doses computed for total 40 c.c. of original solution.....	266,000 to 267,000	800	o	266,000 to 267,000
Percentage of hemolysin in each preparation.....	100	0.3	0.0	100

* Reckoned on the basis of the original aqueous venom solution.

in almost complete amount, from 19 to 19½ of the original 20 c.c. being recovered. Neither of the solutions thus separated differed in optical appearance from the corresponding solution employed in forming the emulsion other than that the aqueous solution was slightly clearer than that first added. The chloroform solution was perfectly clear. Immediately following the separation each solution was tested quantitatively for its hemolytic action. The results showed that 99.7 per cent of the hemolytic power of the original aqueous venom solution had been transferred to the chloroform lecithin solution, the complementary 0.3 per cent being retained by the aqueous solution. The chloroform solution thus known to contain the lysis was next treated with ether in an attempt to isolate the hemolytic substance. Five volumes of chemically pure ether (water-free, distilled over sodium) were added to the chloroform solution with the result that a fairly abundant flocculent precipitate appeared and gradually settled to the bottom of the container leaving an appreciable amount of lecithin dissolved in the supernatant ether-chloroform mixture. Employing the centrifuge this precipitate

was recovered in a compact mass and washed in five volumes of ether by shaking and centrifugalization for from 10 to 20 times to effect a removal of admixed lecithin. The hemolytic power of the precipitate was then tested and proved to be quantitatively that of the chloroform solution prior to precipitation with ether, namely 99.7 per cent of the hemolytic power of the original aqueous venom solution. To make the method quantitatively more perfect the aqueous venom solution which retained 0.3 per cent of its original hemolytic power was treated a second time with chloroform lecithin and the lysin representing this 0.3 per cent precipitated with ether as above. Thus the hemolytic power of the native venom solution was transferred to the chloroform lecithin solution and from this latter solution a substance, cobra lecithid, was isolated which represented quantitatively (100 per cent) the hemolytic power of the original venom solution. Table 24, p. 215, illustrates the hemolytic values at various stages in the procedure as outlined.

In order to determine whether the treatment with chloroform lecithin modified the native venom as a whole or simply its hemolytic principle, the aqueous solution from which the hemotoxin had been completely removed was tested for its neurotoxic action. It was found that the neurotoxin content of the treated venom was exactly that of the same solution before treatment, showing that the lecithin treatment involved the removal of the hemotoxin only. The following table (Table 25) shows the correspondence in toxicity of the venom for mice (15 gm.) before and after the shaking with the chloroform lecithin solution.

TABLE 25.
COMPARATIVE DETERMINATIONS OF NEUROTOXIN CONTENT OF UNMODIFIED AND
HEMOTOXIN-FREE COBRA VENOM.

AMOUNT OF 0.01 PER CENT SOLUTION VENOM c.c.	MICE 15 GM. WEIGHT	
	A Unmodified Venom	B Hemotoxin-free Venom
0.5.....	+ after 2 hours	after 1 hour
0.35.....	+ " 2½ "	+ " 1½ "
0.25.....	+ " 1½ "	+ " 1½ "
0.05.....	+ " 2½ "	+ " 8 "
0.12.....	+ " 30-40 "	+ " 30-40 "
0.10.....	lived	lived

From this table it is seen that the neurotoxic action of the venom after treatment with chloroform lecithin was quantitatively the same as prior, in each instance the minimal lethal dose being 0.12 c.c. of a 0.01 per cent solution. In other words, the entire neurotoxic principle of the native venom had been retained whereas the entire hemotoxic principle had been removed. This actual separation of the hemotoxin and the neurotoxin into two solutions without the destruc-

tion of either added direct proof to the correctness of Myers'¹ contention that these two toxic principles of cobra venom are distinct.

The method of isolating cobra lecithid above outlined was that first employed and in the earlier stages of the work gave constant results. At a later period in the investigation, however, difficulty was experienced in transforming the total hemotoxin content of the venom into its lecithid. In certain instances even after weeks, the agitation of a given cobra venom solution with lecithin chloroform resulted in the formation of but a relatively small amount of cobra lecithid. Inasmuch as new supplies of material had been necessary in the course of the investigation this contradiction of the earlier results was attributed to differences in the materials used. It was at first assumed that the causal differences were those existing between the various specimens of cobra venom. Test-tube experimentation, however, showed the latter preparations of venom to be comparable in hemolytic power to those at first employed. The cause of the disparity in the results was then sought in differences in the lecithins, it being determined that commercial preparations of this substance vary markedly, and that a chief point in the variation is the acidity of the product. The effect upon lecithid formation of alkali neutralization of the acid by-products of lecithin decomposition was tested. It was found that the acidity of these by-products inhibits the formation of cobra lecithid and that by the neutralization of the by-products a constant and complete yield of the lecithid could be obtained. This effect of alkali neutralization upon the lecithid production is illustrated in the following experiment (Table 26).

Four hundred c.c. of a 0.5 solution of cobra venom in water were shaken four weeks with 600 c.c. of 20 per cent lecithin chloroform solution. The resulting emulsion was centrifugalized and from the chloroform portion a small sample was removed and tested for its hemolytic action. The mixture was again emulsified and sufficient NaOH was added to half neutralize its acidity as determined by titration. The emulsion was shaken continuously for two days, at the end of which period an amount of HCl equivalent to the NaOH previously used was added,² the mixture then centrifugalized, and a second test of the chloroform solution made for its hemolytic power.

The small amount of chloroform lecithid solution removed at each of the two periods for testing was so diluted that it represented a 0.005 per cent solution relative to the native venom employed. The hemolytic power was tested by the addition of decreasing amounts of this dilution to 1 c.c. of a 5 per cent suspension of goat's cor-

¹ *Jour. Path. and Bact.*, 1900, 6, p. 415.

² For the elimination of the salts of the fatty acids formed.

puscles. As a control the lytic action was compared with the hemolysis produced by a 0.005 per cent solution of native venom with the same blood unit plus 0.1 c.c. of a 0.1 per cent lecithin solution. The tabulated results are as follows:

TABLE 26.

AMOUNT OF 0.005 PER CENT SOLUTION C.C.	HEMOLYSIS OF 1 C.C. OF A 5 PER CENT SUSPENSION OF GOAT ERYTHROCYTES		
	A Lecithid in Chloroform Solution after Four Weeks' Shaking without Alkali	B Lecithid in Chloroform Solution after Subsequent Two Days' Shaking with Alkali	C Native Venom Comple- mented with Lecithin
1.0.....	complete	complete	complete
0.75.....	"	"	"
0.5.....	"	"	"
0.35.....	marked	"	"
0.25.....	slight	"	"
0.15.....	trace	"	"
0.1.....	o	"	"
0.075.....	o	"	"
0.05.....	o	medium	medium
0.035.....	o	trace	trace
0.025.....	o	faint trace	faint trace
0.015.....	o	o	"
0.01.....	o	o	"

This experiment demonstrated clearly that the alkali neutralization of the acid by-products contained in the venom lecithin mixture removed an inhibition to cobra lecithid formation. Whereas after four weeks' constant shaking but a small portion (approximately 15 per cent) of the hemolytic principle of the venom was found in the chloroform solution, a much shorter time (two days) subsequent to the addition of the alkali sufficed to complete the total transfer of the hemolytic power from the aqueous to the chloroform solution. Therefore taking into account the inhibiting action of acid thus demonstrated the explanation of the inconstant yield of cobra lecithid at times experienced appeared to be in the acidity of certain preparations of lecithin used. At all events the recognition of the inhibiting action of an acid reaction pointed to a modification of the earlier method of lecithid production, which modification resulted in a process giving without fail a 100 per cent yield of cobra lecithid. This process was elaborated as follows:

In order to reduce the time element to a minimum one half neutralization of the acidity of the venom lecithin mixture was effected immediately following rapid emulsification. At once it became apparent, however, that in all such emulsions an increase in acidity accompanied the progress of lecithid production. This constant

increase in acidity, tending to delay lecithid formation, indicated a systematic addition of alkali at subsequent intervals. Therefore immediately after the first neutralization ($\frac{1}{2}$) the emulsion was shaken for two hours, its acidity then determined, and this acidity one half neutralized; it was again shaken for two hours, and then one half neutralized, and the procedure of shaking, titration, and one half neutralization repeated until all signs of increasing acidity ceased.

By employing this system of alkali addition it was possible to effect even in the most unfavorable instances a complete yield of cobra lecithid within a single day. In illustration of the details of the method the following experiment is given (including Table 27).

Four hundred c.c. of a half per cent aqueous solution of cobra venom were shaken vigorously with 400 c.c. of a 20 per cent lecithin chloroform solution. From the resulting emulsion 5 c.c. were then withdrawn and the acidity of this sample titrated with $\frac{1}{10}$ normal NaOH.¹ For this neutralization $3\frac{1}{2}$ c.c. of $\frac{1}{10}$ normal NaOH was

TABLE 27.

AMOUNT OF EACH SOLUTION	HEMOLYSIS OF 1 C.C. 5 PER CENT SUSPENSION OF GOAT ERYTHROCYTES BY:				
	A Aqueous Portion (0.05 per cent)*		B Chloroform Portion (0.005 per cent)*		C Native Cobra Venom (0.005 per cent)+ 0.1 c.c. 0.1 per cent Lecithin
	1 Without Lecithin	2 +0.1 c.c. 0.1 per cent Lecithin	1 Without Lecithin	2 +0.1 c.c. 0.1 per cent Lecithin	
c.c.					
1.0.....		faint trace	complete	complete	complete
0.75.....		" "	"	"	"
0.5.....		" "	"	"	"
0.35.....		" "	"	"	"
0.25.....		" "	"	"	"
0.15.....		" "	"	"	"
0.1.....		" "	"	"	"
0.075.....		" "	"	"	"
0.05.....		" "	marked	marked	almost complete
0.035.....		" "	medium	medium	medium
0.025.....		" "	trace	trace	trace
0.015.....		" "	faint trace	faint trace	faint trace
0.01.....		" "	"	"	"

* The concentration of these solutions is reckoned on the basis of the dried venom used in the original aqueous solution.

required. To the bulk of the emulsion was then added 28 c.c. of normal NaOH or one half the amount of NaOH computed for complete neutralization. After continued shaking of the emulsion for two hours, 5 c.c. were again withdrawn and the acidity determined—2.6 c.c. of a $\frac{1}{10}$ normal NaOH, being required in the titration, indicating the addition of 21 c.c. of normal NaOH to the main bulk of the emulsion for its half

¹ Titration was performed by mixing 5 c.c. of the emulsion with 5 c.c. of amyl alcohol plus 10 c.c. of ethyl alcohol plus three drops of a saturated alcohol phenolphthalin solution.

neutralization. Shaking was resumed for two hours, the then determined acidity of 5 c.c. being 1.9 c.c. of a $\frac{1}{10}$ normal NaOH solution, indicating the addition of 15 c.c. of normal NaOH for one half neutralization of the bulk. Shaking was again continued for two hours, the acidity of 5 c.c. determined to be 1.2 c.c. of a $\frac{1}{10}$ normal NaOH and for half neutralization 10 c.c. NaOH normal were added. Again, shaking was continued for two hours, the acidity for 5 c.c. requiring 1.2 c.c. of $\frac{1}{10}$ normal NaOH solution for neutralization and 10 c.c. of normal NaOH were added for half neutralization. After two hours more of continuous shaking the acidity for 5 c.c. required but 0.6 c.c. of $\frac{1}{10}$ normal NaOH solution for neutralization. In other words the acidity had made no gain during the last period of shaking. An amount of HCl (=84 c.c. normal HCl) equivalent to the total amount of NaOH used up to this point was then added and the emulsion again shaken for two hours. On the following morning the emulsion was centrifugalized and the hemolytic action of the aqueous and chloroform portions determined as indicated in Table 27.

The table just given shows that the entire hemolytic power of the native venom solution was transferred to the chloroform solution and that the lecithid there formed existed as a complete lecithid, that is, one whose lytic action was not increased by the addition of free lecithin.

To isolate the lecithid in substance the 380 c.c. of the chloroform solution recovered in the above procedure were freed from all traces of water by six successive treatments with sodium sulfate and the lecithid then precipitated by the addition of 10 volumes of water-free ether. To effect a complete precipitation the mixture was allowed to stand at -12°C . The resulting white precipitate was washed six times with ether and dried three days *in vacuo* over phosphoric anhydride, sulfuric acid, and paraffin. There resulted 12.4 gm. of substance, somewhat waxy in consistency. This preparation which is comparable to the earliest specimens of cobra lecithid described by me was further purified by dissolving it in 124 c.c. of ethyl alcohol (10 per cent) and by reprecipitation with 10 volumes of ether at -12°C . The resulting precipitate was centrifugalized, washed with ether, and dried to constant weight (10.6 gm.). The lecithid prepared in this manner is snow white and is entirely without the waxy consistency of crude products containing traces of admixed lecithin. This lecithid represents quantitatively the hemolytic action of the chloroform solution from which it was precipitated and therefore that of the original venom solution. Isolated thus in substance the hemolytic action of the lecithid is not in the slightest degree increased by the addition of an excess of free lecithin. This

fact shows the completeness of the reaction afforded by the addition of alkali to the emulsion and contrasts this complete lecithid with other products to be discussed later. The following table in which the concentration of the lecithid solution represents a 0.001 per cent native venom solution indicates the absolute lytic action of the lecithid with and without added lecithin (Table 28).

TABLE 28.
HEMOLYTIC VALUE OF ISOLATED COBRA LECITHID WITH AND WITHOUT ADDED LECITHIN.

COBRA LECITHID C.C.	HEMOLYSIS OF 1 C.C. 5 PER CENT SUSPENSION OF GOAT ERYTHROCYTES BY:	
	a) Cobra Lecithid Alone	b) Cobra Lecithid + 0.1 C.C. 0.1 per cent Lecithin
1.0.....	complete	complete
0.75.....	"	"
0.5.....	"	"
0.35.....	"	"
0.25.....	marked	marked
0.15.....	slight	slight
0.1.....	trace	trace
0.075.....	faint trace	o
0.05.....	o	o

The above table shows that the hemolytic action of the lecithid as isolated is not augmented by added lecithin.

The preparations of cobra lecithid obtained by the last described method in which alkali neutralization is employed are far superior to the earlier lecithid preparations described by me, both from the standpoint of constancy of the yield and the purity of the end product. The chief cause of the difference in the purity of the product is to be found in the fact that in the earlier method of preparation the precipitation of the lecithid from the chloroform solution by ether, carried down a certain amount of admixed lecithin-like substances, the complete removal of which was scarcely possible. Since lecithin itself is ether-soluble these admixed substances which are insoluble in ether may well be considered as hydrates of lecithin. At all events in the last method these admixtures are extensively avoided by the careful dehydration of the chloroform solution with sodium sulfate prior to the ether precipitation of the lecithid. The purification of the precipitate by redissolving in alcohol and reprecipitating with ether is also highly efficient, the admixtures in question appearing never to be precipitated by ether from an alcoholic solution and in this connec-

tion it is also to be noted that the acetone precipitation of lecithin itself varies greatly according to whether the solvent for the lecithin be chloroform or alcohol.

The lecithid as precipitated by ether was found to remain unmodified when allowed to stand under ether, and also to retain its full hemolytic efficiency on drying. The fresh precipitate even when slightly damp with ether was found to be very readily soluble in water giving a perfectly clear light golden solution. Such solutions, however dilute, show a marked tendency to foam when gently agitated. They do not however, even when concentrated, give the biuret reaction. In aqueous solution the cobra lecithid is non-coagulable by heat and retains its hemolytic action on boiling. This thermostability sharply contrasts the hemolysin in this form with the hemolysin in the native venom: Whereas cobra venom in an aqueous solution loses entirely its hemotoxic action when heated for 30 minutes at 100° C., a cobra lecithid solution may be heated six hours at the same temperature without appreciable loss in lytic action. The following table (Table 29) gives the solubilities of cobra lecithid in comparison with those of native cobra venom and of lecithin.

TABLE 29.

Solvent	Cobra Lecithid	Lecithin	Cobra Venom
Water.....	soluble (30° C.)	insoluble	soluble
Benzol.....	soluble	soluble	insoluble
Toluol.....	"	"	"
Chloroform.....	"	"	"
Alcohol.....	"	"	"
Acetone.....	insoluble	insoluble	"
Ether.....	"	soluble	"
Petroleum ether.....	"	"	"

From Table 29 it is to be seen that cobra lecithid possesses distinctive solubilities which differentiate it completely as a chemical entity from the two substances employed in its production. The lecithid in contrast to the native venom is soluble in chloroform, benzol, alcohol, and toluol, whereas in contrast to lecithin the lecithid is insoluble in ether and soluble in water. Dissolved in warm water a portion of the cobra lecithid appears on cooling as a microcrystalline white deposit, the individual crystals being transparent and highly refractive. Upon removal of this precipitate the resulting clear solution again becomes clouded by the further formation of crystals.

In a given instance repeated removals of the crystalline deposit reduced the hemolytic power of a lecithid solution to one-third its original value. The sedimented crystals, on the other hand, when washed in cold water and redissolved in warm water displayed quantitatively the hemolytic power lost from the original lecithid solution.

Cobra lecithid possesses no general toxicity. The absence of such an action would be expected from the fact previously stated that the neurotoxin of the aqueous venom solution is not transferred to the lecithin chloroform solution when shaken with the latter. Large quantities of the pure lecithid injected into animals show no lethal action. This fact which I have previously reported¹ has been more recently challenged by Morgenroth and Carpi.² These authors find that preparations of cobra lecithid which they isolated possess a lethal action and this action they refer to the presence of a neurotoxin lecithid formed coincidently with the hemotoxin lecithid (cobra lecithid) and supposedly admixed therewith.

It appears to me that the difference in the results obtained by Morgenroth and Carpi and by myself is to be referred to a difference in the preparation of the lecithids tested, and in the experiments of Morgenroth and Carpi I see no proof of the actual formation of a lecithid of the neurotoxin of cobra venom.

A portion of the lecithid employed by Morgenroth and Carpi was prepared by a method essentially different from that used by me in isolating the non-toxic lecithids, and one which is inapplicable where a refined product is desired. The method referred to is the so-called "alcohol method"³ which I devised early in the lecithid work as a makeshift to be used in those instances where the amount of material was very small and where no attempt was to be made to completely isolate the lecithid but to determine only its presence. Morgenroth and Carpi misapplied this method in attempting the isolation of a pure lecithid by its use. Reference to the method shows that a mixture of venom and lecithin in 50 per cent methyl alcohol is precipi-

¹ *Berl. klin. Wchnschr.*, 1903, 42, p. 956.

² *Biochem. Ztschr.*, 1907, 4, p. 248.

³ In detail the method is as follows: 1 c.c. of a four per cent aqueous solution of venom is added to 1 c.c. of a 20 per cent solution of lecithin and the mixture allowed to stand several hours at 37° C., being subjected to occasional shaking. To the mixture is then added 10 c.c. of ethyl alcohol and the resulting albuminous precipitate is removed. The resulting filtrate is then precipitated with an excess of ether, the precipitate containing the venom lecithid. Cf. Kyes, *Berl. klin. Wchnschr.*, 1903, 42, p. 963.

tated by the addition of ethyl alcohol and that the filtrate thus obtained is then precipitated with ether for a yield of lecithid. In such a procedure it is far from impossible that a small amount of native neurotoxin may remain in the water-containing alcohol sufficiently long to be collected with the ether precipitated lecithid. It is significant that Morgenroth and Carpi obtained their most toxic products by this method.

Likewise the preparations of cobra lecithid which Morgenroth and Carpi isolated by the "chloroform method" are not above the suspicion of containing a sufficient admixture of native neurotoxin to account entirely for their lethal action. When in the "chloroform method" the aqueous solution of native venom is shaken with the chloroform lecithin solution, the emulsion formed may be so fine that traces of the aqueous solution may remain in the latter despite the most persistent centrifugalization, and the substances thus retained appear falsely as chloroform soluble. The precipitation of such chloroform lecithin with ether will then yield a crude lecithid admixed with a certain amount of native venom constituents including neurotoxin. To guard against this occurrence in preparing pure lecithid the chloroform solution after separation by centrifugalization must be thoroughly treated with a dehydrating agent and again centrifugalized.¹ Inasmuch as Morgenroth and Carpi appear to have omitted this step in their procedure, the lecithids which they obtained even with the chloroform method must be considered as somewhat unrefined products, the toxicity of which may well be referred to a content of native neurotoxin.

The fact cited by Morgenroth and Carpi that the admixed neurotoxin is somewhat more thermostabile than native neurotoxin as usually tested is no proof whatsoever that the neurotoxin is present as a lecithid. Sachs and I have emphasized the fact that the heat resistance of venom constituents is greatly modified by the nature of the solvent in which the venom is tested. That the same neurotoxin in a solution of cobra lecithid should show a heat resistance greater than that which it exhibited when tested in water would not be surprising. The same comment applies also to the modification of toxic symptoms which Morgenroth and Carpi cite as an

¹ Cf. use of sodium sulfate in method as given on p. 220.

indication of the transformation of the neurotoxin into a lecithid. These differences are quite as easily explained by the differences in absorption and distribution of the native neurotoxin when injected in a simple aqueous solution and when injected in a solution of cobra lecithid. Then too the reaction of the admixed neurotoxin in Morgenroth and Carpi's preparations with Calmette's serum argues rather against than for its existence in a modified form. According to these authors the admixed neurotoxin reacts with cobra antitoxin exactly the same as does the native neurotoxin of cobra venom.

Finally, however, it is certain that the hemolytic cobra lecithid when properly isolated by the chloroform method contains no traces of a neurotoxic lecithid. Relatively large quantities of the cobra lecithid in aqueous solution produce no general toxic symptoms when injected into animals. Thus an amount of lecithid sufficient to dissolve 200 c.c. of mouse blood was injected into a mouse of 15 gm. weight and produced no symptoms other than an infiltration at the site of inoculation. Likewise 10 c.c. of a one per cent solution of the lecithid injected subcutaneously into a rabbit of 1,750 gm. weight produced no general symptoms but only a circumscribed infiltration of the ventral abdominal wall. More recently also I have injected subcutaneously 1 c.c. of a 10 per cent lecithid solution into each of a series of seven mice without a single lethal result. In view of these facts I can only conclude as previously, that cobra lecithid when actually isolated is non-toxic, and that the results observed by Morgenroth and Carpi may well be referred to admixture of native neurotoxin with the lecithid which they employed.

In addition to its distinctive solubilities and its thermostability cobra lecithid displays in its biological reactions differences which also contrast it with native venom. It is not surprising that the cobra lecithid, formed as it is in the presence of an excess of lecithin, should possess a hemolytic power entirely independent of the lecithin content of the cell upon which it is acting. It will be recalled that the hemolytic action of native cobra venom is in itself limited to those species of erythrocytes which afford intracellular lecithin for the activation of the venom hemotoxin, and that the species of erythrocytes on this basis fall into two classes, the susceptible and the non-susceptible. Not so with the cobra lecithid. The lecithid dissolves all erythrocytes

regardless of species, and to approximately the same extent. The average dose of lecithid necessary for the hemolysis of 1 c.c. of a 5 per cent suspension of erythrocytes is for all species the proportional amount of lecithid formed from 0.003 mgm. of dried venom. It is to be noted that this amount of venom corresponds to the minimal lytic dose required for hemolysis in the test-tube experiments where an excess of extracellular lecithin is used.

A second feature of the lecithid hemolysis which differs from the hemolysis by the native venom is the rapidity of the reaction. When cobra venom is allowed to act on susceptible blood corpuscles a distinct incubation period always elapses before hemolysis occurs. With minimal doses of the native venom the time required for complete hemolysis is from 12 to 18 hours (two hours at 37°, remainder at 8° C.). By the employment of relatively large doses with highly susceptible corpuscles (guinea-pig) the incubation period may be shortened to from 10 to 30 minutes, but even in extreme cases this interval never entirely disappears. Likewise when the minimal lytic dose of venom is activated for non-susceptible corpuscles (goat) by the minimal amount of extracellular lecithin, there must elapse from 16 to 20 hours before the hemolysis is complete. Larger doses of venom and lecithin tend to decrease the incubation period but even under these conditions a distinct incubation period is always to be observed with the native venom. In contrast to this incubation period constantly exhibited by native venom the rapidity of the hemolysis effected by cobra lecithid is marked. In the case of concentrated solutions of the latter the hemolysis is instantaneous. Even where dilute solutions are employed the delay is slight, the maximum being from 15 to 20 minutes. With corresponding doses of the native venom and of the lecithid, where the incubation period with the native venom must be expressed in hours, that with the lecithid is a matter of as many minutes.

The difference in the time required for hemolysis by the venom and its lecithid is of especial importance inasmuch as it shows that the incubation period observed with the native venom is not the time required for the dissolution of the cell by the lysin but rather that it is the time required for the elaboration of the complete lysin from the native hemotoxin and its activating substance—the complete lysin

(lecithid) once formed, there is no delay due to the gradual action of the toxophore group of the toxic complex but the hemolysis is immediate. With this appreciation of the exact nature of the incubation period, the difference between the length of the period, where large and where small doses of venom and lecithin are used, finds its logical chemical explanation in a difference in the rate of lecithid formation in concentrated and in dilute solutions of the substances involved in its production.

An additional important point in which the hemolysis by venom lecithid differs from that produced by the native venom is that whereas the specific antibody (Calmette's antiserum) for cobra venom markedly inhibits hemolysis by the native venom, it possesses no corresponding inhibiting action for the lecithid of the same venom. On the other hand the inhibiting action of cholesterin on native venom hemolysis occurs also with the lecithid hemolysis as shown in the following experiment (Table 30):

TABLE 30.
CHOLESTERIN INHIBITION OF COBRA LECITHID HEMOLYSIS.

AMOUNT OF CHOLESTERIN SOLUTION* c.c.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES
	1½ Lytic Doses of Cobra Lecithid
0.1.....	0
0.075.....	0
0.05.....	0
0.035.....	0
0.025.....	0
0.015.....	almost 0
0.01.....	trace
0.0075.....	slight
0.005.....	medium
0.0035.....	marked
0.0025.....	almost complete
0.0015.....	complete
0.001.....	complete

* The cholesterin solution was made by adding 1 c.c. of a hot saturated methyl alcohol solution of cholesterin to 9 c.c. of 0.85 per cent NaCl solution.

The introduction of the technic of ether precipitation from alcohol into the process of cobra lecithid isolation so far enhanced the value of the method for obtaining a pure end product as to allow trustworthy chemical analyses. In these analyses the co-operation of Willstätter and Lüdecke and of von Braun was enjoyed and independent determinations of several preparations of complete lecithid

showed the preparations to consist of one and the same substance. The elementary analysis for nitrogen and phosphorus at the hands of Lüdecke[†] gave the following values:

- a) 0.2935 gm. = 7.25 c.c. Nitrogen
 $\frac{0.3018 \text{ gm.} = 0.0625 \text{ gm. Mg}_2\text{P}_2\text{O}_7}{\text{N} = 2.73 \text{ per cent; P} = 5.76 \text{ per cent}}$
- b) 0.1994 gm. = 4.99 c.c. Nitrogen
 $\frac{0.1859 \text{ gm.} = 0.0403 \text{ gm. Mg}_2\text{P}_2\text{O}_7}{\text{N} = 2.8 \text{ per cent; P} = 6.03 \text{ per cent}}$

The corresponding analysis of another preparation by von Braun gave the following results:

$$\begin{array}{l} 0.169 \text{ gm.} = 3.89 \text{ c.c. Nitrogen} \\ 0.5118 \text{ gm.} = 0.1045 \text{ gm. Mg}_2\text{P}_2\text{O}_7 \\ \text{N} = 2.84 \text{ per cent; P} = 5.56 \text{ per cent} \\ \text{Hydrogen} = 10.92 \text{ per cent} \\ \text{Carbon} = 59.07 \text{ per cent} \end{array}$$

The values for N and P are seen to agree closely in the several determinations and these values correspond with those of a lecithin minus a fatty acid. Thus for a monostearic lecithin Lüdecke gives:

$$\text{N} = 2.59 \text{ per cent; P} = 5.73 \text{ per cent;}$$

and for a monopalmitic lecithin:

$$\text{N} = 2.74 \text{ per cent; P} = 6.06 \text{ per cent.}$$

The important evidence was thus obtained that in the lecithid formation there is a splitting off of a fatty acid radicle, a fact previously indicated by the increase in acidity of the aqueous chloroform emulsion in the method of preparation above outlined. Further in accord with this finding is also the determination by Lüdecke of the presence of a free fatty acid in the ether employed in precipitating the lecithid from the original chloroform solution.

The direct chemical analyses corroborate, then, the evidence furnished by the distinctive solubilities and biological reactions of cobra lecithid, namely, in showing that this product is a newly formed chemical substance resulting from a reaction between venom and lecithin in which there occurs a splitting of a fatty acid radicle from the latter substance.

[†] Cf. K. Lüdecke, Dissert., Munich, 1905.

The question now logically arises as to whether the lecithid is a synthetic product resulting from the combination of a venom and lecithin quota or whether it represents simply a split product of lecithin resulting from a lipolytic action of the cobra venom: The elementary chemical analyses given above offer no evidence for the determination of this point. The amount of the venom constituent in the lecithid, if such there be, is so slight in comparison with the lecithin quota that its presence is not determined by such chemical methods. On the other hand, as I have elsewhere stated, there are data which strongly favor the assumption that the lecithid is a synthetic product.

Among these data is the fact that the lecithid as a hemolysin possesses an extreme toxicity comparable to that which is constantly noted with the true toxins but which can hardly be ascribed to a monostearyl-lecithin. A proof of greater value, however, and one deserving special emphasis from the biological point of view, is the fact that immunization with cobra lecithid yields an antibody which acts not only upon the lecithid to effect its neutralization but also upon the native cobra venom. The production of such an antibody shows at the same time that the lecithid is a true toxin and also that it contains a quota of the native venom sufficient to stimulate the production of an antibody specific for such native venom.

A series of rabbits were immunized by the subcutaneous injection of increasing doses of cobra lecithid. Prior to the first injection the degree of the normal inhibiting action of the serum (common to all rabbits) was determined for each animal and it was further determined in each instance that this normal inhibiting action was removed by heating the serum for $\frac{1}{2}$ hour at 64° – 65° C. The specific neutralizing action of the immune serum was tested quantitatively at various stages in the immunization, the destruction of the non-specific inhibiting action being first effected by heating as above indicated. Not only were the resulting immune sera highly efficient in neutralizing cobra lecithid, but their progression in antibody content was clearly demonstrated. The following table (Table 31) indicates the efficiency of the serum of the same rabbit at four different points during its immunization.

From this table it is seen that a serum which at first had no

specific neutralizing action for the lecithid gradually attained this power as a result of successive inoculations with the lecithid until 0.025 c.c. of the serum sufficed to neutralize completely the lecithid.

TABLE 31.
ANTI-COBRA LECITHID.

AMOUNT OF SERUM ADDED C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES ADDED TO EACH TUBE AFTER SERUM AND LECITHID (2 LYTIC DOSES) HAD STOOD $\frac{1}{2}$ HOUR AT 37° C.				
	I Serum Drawn Prior to Inoculation	Serum Drawn at a Period after Inoculation with:			
		II 60 c.c. 0.1 per cent Lecithid*	III 85 c.c. 0.1 per cent Lecithid	IV 100 c.c. 0.1 per cent Lecithid	V 200 c.c. 0.1 per cent Lecithid
0.75.....	complete	o	o	o	o
0.5.....	"	almost o	o	o	o
0.35.....	"	trace	o	o	o
0.25.....	"	slight	o	o	o
0.2.....	"	medium	o	o	o
0.15.....	"	complete	o	o	o
0.1.....	"	"	o	o	o
0.075.....	"	"	almost o	o	o
0.05.....	"	"	slight	o	o
0.035.....	"	"	almost complete	almost o	o
0.025.....	"	"	complete	slight	o
0.02.....	"	"	"	medium	almost o
0.015.....	"	"	"	marked	trace
0.01.....	"	"	"	almost complete	slight
0.0075.....	"	"	"	"	medium
0.005.....	"	"	"	complete	"
0.0035.....	"	"	"	"	marked
0.0025.....	"	"	"	"	almost complete
0.002.....	"	"	"	"	complete

* The lecithid used for inoculation was in all instances a complete lecithid which contained neither native venom nor admixed lecithin. The lecithid was boiled prior to inoculation.

The following experiment on the other hand shows the neutralizing action of the same immune serum for native cobra venom (Table 32).

This table shows that the anti-cobra lecithid serum, in addition to neutralizing the lecithid, neutralizes the hemotoxin of the native venom; and not only to an equal but to a greater extent. The reasons for this quantitative difference will be discussed in a later chapter, but at this point it is to be emphasized that the specific antibody obtained by immunization with cobra lecithid reacts with native cobra venom, indicating that the lecithid contains a side chain derived from the venom.¹

From the standpoint of physical chemistry also the conception of the lecithid as a synthetic product receives substantial support.

¹ For a consideration of the work of von Dungern and Coca in this connection (*Munch. med. Wchnschr.*, 1907, 54, p. 2317; and *Biochem. Ztschr.*, 1908, 12, p. 407) see section VI, p. 272.

Determinations at the hands of Biltz of Clausthal show that the molecular weight of cobra lecithid in chloroform is between 2,000 and 3,000. The molecular weight of lecithin itself is variously determined as between 777 and 807, while Willstätter computes 541 for mono-stearyl-lecithin. Thus it appears that the lecithid cannot be a split product of lecithin but that a venom constituent with lecithinophile groups must unite many monostearyl-lecithins to produce a body of high molecular weight.

TABLE 32.

AMOUNT OF SERUM ADDED	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES			
	I. Control Normal Serum (Heated $\frac{1}{2}$ Hour 64° C.)		II. Anti-Cobra Lecithid Serum (Heated $\frac{1}{2}$ Hour 64° C.)	
	With Cobra Lecithid*	With Cobra Venom	With Cobra Lecithid	With Cobra Venom
C.C.				
0.1.....	complete	complete	o	o
0.075.....	"	"	o	o
0.05.....	"	"	o	o
0.035.....	"	"	o	o
0.025.....	"	"	o	o
0.02.....	"	"	almost o	o
0.015.....	"	"	trace	o
0.01.....	"	"	slight	o
0.0075.....	"	"	medium	o
0.005.....	"	"	"	o
0.0035.....	"	"	marked	slight
0.0025.....	"	"	almost complete	marked
0.002.....	"	"	complete	almost complete
0.0015.....	"	"	"	complete
0.001.....	"	"	"	"

* Twice the lytic dose employed in case of each lysin. Venom complemented with lecithin (0.2 c.c. 0.1 per cent).

In view of the results above outlined, I am at present of the opinion that in the formation of cobra lecithid there is a splitting off of a fatty acid radicle and a synthesis of the remaining lecithin molecule with a venom constituent.

Thus far the only lecithid discussed has been that formed with cobra venom. If however the activating action of lecithin in general is to be referred to the formation of lecithids, the isolation of such lecithids should be possible in all instances of a true activation. Such is actually the case. From the full series of toxins mentioned in section as III being activated by lecithin, I have prepared and isolated, in substance, typical lecithids. The general characteristics of these lecithids correspond with those displayed by the lecithid of cobra venom. The quantity of lecithid formed in each instance bears the

same relation to the hemolytic power of the venom, as seen in the case of cobra venom. Thus those venoms which show the same hemolytic power as cobra venom when activated by lecithin in test-tube experiments, produce quantitatively the same yield of lecithid by weight. Further, those venoms (Bothrops and trimeresurus) which showed but a fraction ($\frac{1}{10}$) of the lytic power of the other venoms as tested in the activating experiments, yielded exactly this fraction ($\frac{1}{10}$) of lecithid. Nor is the lecithid formation confined to the snake venoms. Scorpion poison formed quantitatively the quota of lecithid corresponding to its hemolytic power when activated by an excess of lecithin in the test-tube experiments.

The lecithids thus far discussed are those prepared in the presence of an excess of lecithin and are the so-called complete lecithids. In these preparations the lecithinophile groups of the venom hemotoxin are fully saturated. Of equal importance in the analysis of the relation of the venom and the lecithin constituents, however, is the nature of the compounds formed in the presence of relatively small amounts of lecithin—the so-called incomplete lecithids. To obtain products of this sort experimental conditions were so modified that for each gram of cobra venom two grams of lecithin were employed. A one per cent solution of cobra venom was shaken with a 10 per cent solution of lecithin in chloroform as in the perfected method described for the production of complete lecithids, until the production of acid ceased. Provisional tests showed that in this procedure the hemolytic power was not transferred to the chloroform but was retained entirely by the aqueous solution. The complete separation of the resulting viscous emulsion, however, incurred great difficulties. Continued centrifugalization was insufficient and only after many modifications of experimentation was it possible to accomplish this end. Finally however, it was determined that the addition of abundant alcohol and ether served as a simple means for separating the aqueous and chloroform portions of the emulsion. The addition of the ether and alcohol so diluted the chloroform solution and decreased its specific gravity that a spontaneous separation occurred with the aqueous portion subnatant. The details of this procedure are given below:

One thousand five hundred and seventy c.c. of one per cent cobra venom were added to 315 c.c. of 10 per cent chloroform lecithin and shaken until titration showed

no increase in acidity. During the shaking 68 c.c. of n/NaOH were used and at its completion the corresponding 68 c.c. of n/HCl were added. The resulting volume at this stage was 1,993 c.c. (2,000 in round numbers). The required proportion of ether and alcohol necessary to effect separation of the emulsion was then added: ether, one volume (2,000 c.c.); alcohol, $\frac{2}{3}$ volume (800 c.c.). The mixture was then vigorously shaken and allowed to stand over night in a separating funnel. The next morning the aqueous portion was found distinctly separated from the ether-alcohol diluted chloroform portion and was subnatant. The aqueous portion contained a sedimented precipitate. The aqueous portion with its sediment was withdrawn and the sediment removed by centrifugalization. The decanted aqueous portion was then placed at -12°C . with the result that a further precipitate appeared which was removed by centrifugalization. The decanted aqueous portion was again placed at -12°C . for several days, resulting in the further formation of a precipitate from which the supernatant aqueous portion was removed by decantation and then filtered. The several yields of the precipitate were combined, and the aqueous solution (Solution X) showing no further precipitate at -12°C . was preserved for manipulation as hereafter given.

The sedimented precipitate obtained in the above procedure was many times washed with an excess of 30 per cent alcohol at -12°C ., preliminary experiments having shown the precipitate to be insoluble under such conditions. The purified product was then regained by

TABLE 33.

AMOUNT OF EACH SOLUTION C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES		
	Substance I (0.01 per cent Solution)		Cobra Venom (0.01 per cent) + 0.1 c.c. 0.1 per cent Lecithin
	a) Without Lecithin	b) + 0.1 c.c. 0.1 per cent Lecithin	
1.0.....	complete	complete	complete
0.5.....	medium	"	"
0.25.....	trace	"	"
0.15.....	o	"	"
0.1.....	o	"	"
0.05.....	o	"	"
0.025.....	o	almost complete	"
0.015.....	o	marked	"
0.01.....	o	—	"
0.005.....	o	—	"
0.0025.....	o	—	almost complete
0.0015.....	o	—	marked
0.001.....	o	—	medium
0.....	o	o	o

centrifugalization, spread on a porcelain plate, and dried *in vacuo* over sulphuric acid. The product weighed 3.35 gm. This substance, designated as Substance I, reacted slightly acid when suspended in water, and was readily dissolved in warm water upon the addition of a trace of alkali or acid, without reprecipitating on cooling. The clear, slightly alkaline solution gave the biuret reaction and numerous protein precipitation reactions: picric acid, tannic acid, nitric acid,

etc. The addition of an alcoholic cadmium chloride solution caused a voluminous precipitate. Tested for its hemolytic power, Substance I showed the values given in Table 33, p. 233.

This table shows that the hemolytic action of Substance I was increased twenty fold by activation with lecithin but even at that represented but one-tenth the strength of native cobra venom. Consideration of the fact also that but 3.35 gm. of Substance I was obtained as against 15.7 gm. of native venom used in the process showed that the total hemotoxin regained in Substance I was but $\frac{1}{47}$ the total hemotoxin employed. The remaining hemotoxin was therefore sought in the aqueous solution (Solution X) from which Substance I had been originally sedimented and was there found. The hemolytic action of Solution X, as displayed by a small portion withdrawn for the test, is given below (Table 34):

TABLE 34.

QUANTITY IN C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES		
	Solution X (0.01 per cent)*		Cobra Venom (0.01 per cent) + 0.1 C.C. 0.1 per cent Lecithin
	a) Without Lecithin	b) + 0.1 C.C. 0.1 per cent Lecithin	
1.0.....	complete	complete	complete
0.75.....	"	"	"
0.5.....	"	"	"
0.35.....	"	"	"
0.25.....	almost complete	"	"
0.15.....	trace	"	"
0.1.....	o	"	"
0.075.....	o	"	"
0.05.....	o	"	"
0.035.....	o	"	"
0.025.....	o	"	"
0.015.....	o	"	"
0.01.....	o	"	"
0.0075.....	o	"	"
0.006.....	o	"	"
0.005.....	o	almost complete	almost complete
0.0035.....	o	marked	marked
0.0025.....	o	medium	medium
0.0015.....	o	slight	slight
0.001.....	o	trace	trace
0.....	o	o	o

* The volume of Solution X as recovered was approximately 2,000 c.c. which in comparison with the original venom solution (1,570 c.c.) represented a $\frac{1}{3}$ dilution. A portion of Solution X further diluted 1:7 (8X) represented therefore a 0.1 per cent venom solution and again diluted ten times as used above, a 0.01 per cent venom solution.

This table shows that the hemolytic action of Solution X was increased 58 fold by lecithin activation and that the total amount of hemolysin contained was approximately that of the original venom

employed. The bulk of Solution X was next treated as follows: By preliminary experiments with small quantities it was determined that by the addition of phenol, an oily, golden-brown diffuent sediment could be obtained from Solution X and that such sediment contained its hemolytic principle in a concentrated form; and further that this separation was best effected when a $\frac{1}{2}$ volume of 5 per cent phenol was added at the temperature of melting ice. On this basis a considerable portion of Solution X was treated in the following manner:

Nine hundred c.c. of Solution X were shaken vigorously with 450 c.c. of a 5 per cent phenol solution and the mixture removed to a funnel with a sub-attached tube, placed in ice. On the following day the oily sediment was drawn off, the amount recovered being 14 c.c., and rendered clear by the addition of 10 c.c. of alcohol and centrifugalization. The resulting clear fluid was then diluted to 200 c.c. by the addition of alcohol. From this preparation 1 c.c. was immediately taken and diluted with 35 c.c. of physiological salt solution, this giving a dilution which computed relative to the original one per cent venom solution corresponded to a 0.1 per cent venom solution and is designated as "0.1 per cent relative." The hemolytic action of this solution is given in Table 35.

TABLE 35.

QUANTITY IN C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES + 0.1 C.C. 0.1 PER CENT LECITHIN	
	Phenol Sedimented Oil (0.1 per cent rel.)	Cobra Venom (0.1 per cent)
0.0001.....	complete	complete
0.000075.....	marked	"
0.00005.....	medium	"
0.000035.....	"	"
0.000025.....	"	marked
0.000015.....	slight	medium
0.00001.....	trace	"
0.....	0	0

Table 35 shows that whereas Solution X contained approximately the full amount of hemotoxin contained by the corresponding venom solution, the oily sediment recovered from Solution X by treatment with phenol contained but $\frac{1}{3}$ this amount. In this process therefore a relatively large amount of the hemolysin was destroyed or else evaded sedimentation. But by treating a second portion of Solution X (620 c.c.) as above, an additional 12 c.c. of the oily sediment was obtained and the combined products sufficed for the analyses hereafter referred to. To obtain the hemolysin content of the oily substance, the latter product was placed at -12°C . in a quantity of alcohol amounting to 15 volumes. There resulted a precipitate

which was collected by centrifugalization and washed with alcohol. This product when dried *in vacuo* weighed 0.514 gm. and the hemolytic power of a 0.1 per cent solution of the same is displayed in Table 36.

TABLE 36.

AMOUNT IN C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES		
	Substance II (0.1 per cent)		Cobra Venom 0.1 per cent +0.1 c.c. 0.1 per cent Lecithin
	Without Lecithin	+0.1 c.c. 0.1 per cent Lecithin	
1.0.....	faint trace	complete	complete
0.5.....	" "	"	"
0.25.....	o	"	"
0.1.....	o	"	"
0.05.....	o	"	"
0.025.....	o	"	"
0.01.....	o	"	"
0.005.....	o	"	"
0.0025.....	o	"	"
0.001.....	o	"	"
0.0005.....	o	"	"
0.00025.....	o	"	marked
0.0001.....	o	"	medium
0.00005.....	o	"	slight
0.000025.....	o	medium	"
0.00001.....	o	"	faint trace
0.....	o	o	o

This table shows that Substance II although not hemolytic by itself even in large doses was when activated by lecithin tenfold as hemolytic as native cobra venom. This fact itself showed the preparation to be free of lecithin. Notwithstanding the extreme hemotoxic action of Substance II however, the total hemolytic power of the 0.514 gm. isolated represented but about $\frac{1}{3}$ the hemolytic power of the quantity of Solution X from which the oil was derived, and therefore but $\frac{1}{3}$ of the corresponding amount of the original venom solution.

The filtrate resulting from the precipitation of Substance II from the oil by alcohol was next precipitated with an excess of ether and the sediment isolated—Substance III. This precipitate was voluminous and gelatinous, and soluble in water. In an aqueous solution it was shaken vigorously with ether, regained by sedimentation, and dried *in vacuo* to constant weight. The end product was of a light golden-brown color and weighed 2.3 gm. The hemolytic power is given in Table 37.

From this table it is seen that Substance III possessed some hemo-

lytic action without the addition of lecithin but that with the addition of lecithin, the hemolytic action was 133 times increased, then closely corresponding to that of native venom. In other words the 2.3 gm. of Substance III possessed the same hemolytic power as 2.3 gm. of venom. But inasmuch as the corresponding amount of the original venom solution, of which Substance III was a product, contained

TABLE 37.

AMOUNT IN C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES		
	Substance III (0.1 per cent)		Cobra Venom 0.1 per cent +0.1 c.c. 0.1 per cent Lecithin
	Without Lecithin	+0.1 c.c. 0.1 per cent Lecithin	
1.0.....	complete	complete	complete
0.5.....	"	"	"
0.1.....	"	"	"
0.05.....	slight	"	"
0.01.....	o	"	"
0.005.....	o	"	"
0.001.....	o	"	"
0.00075.....	o	"	"
0.0005.....	o	medium	marked
0.00035.....	o	"	"
0.00025.....	o	slight	medium
0.00015.....	o	"	"
0.0001.....	o	"	"
0.....	o	o	o

11.8 gm. of venom, but $\frac{1}{8}$ of its total hemotoxin was isolated as Substance III. In view of the fact that but a very slight hemolytic action was displayed by the aqueous solution derived from Solution X after its treatment with phenol and the removal of the oil, it is evident that in the treatment with phenol a large amount of hemotoxin was either destroyed or so modified as to escape detection. For completeness however about 2,000 c.c. of the aqueous solution above referred to (Solution X) was greatly concentrated by slow distillation *in vacuo* at 50° C. and precipitated with an excess of alcohol. This precipitate, soluble in water, gave the biuret reaction. It was reprecipitated with alcohol and dried *in vacuo*, giving a product (Substance IV) weighing 2.7 gm.¹

A summary of the above process shows that from a venom solution allowed to react with a relatively small amount of lecithin, the follow-

¹ The filtrate resulting from the alcohol precipitation of concentrated Solution X gave a precipitate on the addition of cadmium chloride. This precipitate, when washed with alcohol and dried, weighed 0.4 gm.

ing substances were differentiated on the basis of their varying solubilities and were isolated:

1. Substance I, appearing as a spontaneous precipitate in the aqueous portion after separation of latter from chloroform portion of original emulsion: Representing $\frac{1}{47}$ of the total hemolytic efficiency of the original venom. Amount actually isolated, 3.35 gm.

2. Substance II, precipitated by alcohol from an oily sediment obtained by treating the aqueous solution with phenol: Representing $\frac{1}{3}$ the total hemolytic efficiency of the original venom. Amount actually isolated, 0.154 gm.

3. Substance III, precipitated by ether from the alcoholic filtrate removed from Substance II: Representing $\frac{1}{6}$ the total hemolytic efficiency of the original venom. Amount actually isolated 2.3 gm.

4. Substance IV, precipitated by alcohol from the aqueous solution resulting after removal of oily sediment from same by phenol treatment: Representing non-hemolytic constituents of the original venom. Amount actually isolated, 2.7 gm.

The chemical analyses of these several products I have given in detail in another place,¹ appreciating, however, that the methods used in their isolation are far less efficient in securing fully isolated substances than in the case of the complete lecithids. The results are sufficient, however, to emphasize the fact that when in the production of a lecithid the amount of lecithin used is insufficient to allow the formation of a complete lecithid hemotoxin, lecithin compounds are formed which differ both from the native venom and from the complete lecithid. Substance III is a body which unlike the native venom is soluble in alcohol, and yet this body represents the venom hemotoxin in a modified form, for when activated by lecithin its hemolytic power is tremendously increased by the formation of a complete lecithid. That there is a lecithin quota in this alcohol soluble hemotoxin compound is shown by its 2.1 per cent phosphorus content.

It is not improbable that a series of incomplete lecithids may be formed differing from one another in the number of lecithinophile radicles of the hemotoxin which are occupied with monostearyllecithin. By a method of procedure differing somewhat from that given above but in which also a relatively small amount of lecithin

¹ *Biochem. Ztschr.*, 1907, 4, p. 99.

was employed, I have isolated an incomplete lecithid quite unlike that described above in that it is insoluble in alcohol—a point which serves also to distinguish this latter lecithid from the complete lecithid. On the other hand this lecithid differs from native hemotoxin in that it is non-susceptible to the action of cobra antitoxin (antivenin, Calmette), in this respect coinciding with a characteristic of the complete lecithid. As stated previously, the transformation of the native hemotoxin into the complete lecithid so reduces its affinity for antivenin that this antibody is without inhibiting action upon the complete lecithid. Likewise, the lecithid under discussion, although incomplete, is seen to contain sufficient of the lecithin quota to decrease its affinity for cobra antitoxin to that point where it resists neutralization. The indifference of this incomplete lecithid to Calmette's serum is given in a comparison with native venom in Table 38.

TABLE 38.

AMOUNT OF ANTI- VENOM	1 C.C. 5 PER CENT SUSPENSION OF GOAT ERYTHROCYTES	
	Incomplete Lecithid (3 Lytic Doses) +0.1 c.c. 0.1 per cent Lecithin	Cobra Venom (3 Lytic Doses) +0.1 c.c. 0.1 per cent Lecithin
C.C.		
0.05.....	complete	o
0.025.....	"	o
0.015.....	"	o
0.01.....	"	trace
0.005.....	"	complete
0.....	"	"

From Table 38 it is seen that the incomplete lecithid was not neutralized at all by the same cobra antitoxin which completely neutralized the native cobra hemotoxin. It appears then that this lecithid, though far from being a complete lecithid as shown by the character of its hemolysis and its insolubility in alcohol, nevertheless possessed a sufficient quota of monostearyl-lecithin to decrease its affinity for cobra antitoxin in a way comparable to that observed with the complete lecithid.

Substances similar to the incomplete lecithids just described, I have also isolated from the chloroform lecithin solution resulting from attempts to produce a complete lecithid without the addition of alkali. In such instances there are present, in addition to the small

amount of complete lecithid formed, lipoid-hemotoxin compounds which are soluble in chloroform and in alcohol and which are activated by the addition of lecithin in the test-tube experiments.

The existence of incomplete lecithids as a group is strong proof of the synthetic character of the reaction between the venom hemotoxin and lecithin. The existence of an alcohol soluble substance as in the instance first cited, susceptible to activation by lecithin and possessing a relatively high phosphorus content, denotes a modification of the venom hemotoxin in which a phosphorus-containing radicle of lecithin is synthetically involved.

In view of the facts presented in this section concerning the lecithids and more particularly the cobra lecithids, the following conclusions seem justified by the way of summary. The production and isolation of the complete lecithid shows that the activation of venom hemotoxin by lecithin is essentially a reaction between these two substances resulting in an end product which is the actual lytic agent: The splitting off of a fatty acid from the lecithin in the production of the lecithid together with the total absence both of native venom and of lecithin in the end product shows at once that the reaction is a chemical reaction and a non-reversible chemical reaction: The production of a specific antibody to the lecithid by immunization and the reaction of this antibody with native hemotoxin shows at once that the lecithid is a toxin and that it embraces a constituent of the native venom: The chemical analysis on the other hand shows that the lecithid contains also a lecithin constituent: Taken together these facts indicate that the reaction by which the lecithid is formed is a synthesis, which fact is further supported by the occurrence of intermediate products, the incomplete lecithids differing both from the native hemotoxin and from the complete lecithid, but possessing some characteristics of each.

From a general physiological point of view these data concerning the lecithids merit consideration. The transformation of the native venom hemolysins into the fat-soluble lecithids suggests the possibility that many neurotropic toxins, which themselves are insoluble in the tissues of the nervous system, may within the host combine with various lipoids (lecithin, cholesterin, etc.), and thus assuming a lipoid character become the true neurotropic toxins soluble within

the cell. The existence of the incomplete lecithids suggests moreover that for such a transformation the necessary supply of lipoids in the host need not be so great, for in this instance but a relatively small amount of lecithin is required to give the protein constituent certain solubilities of the lipoids.

V.

SERUM ACTIVATION.

The action of normal serum favoring venom hemolysis was first described by Stephens¹ in 1898. This author showed by quantitative experiments that erythrocytes not otherwise dissolved by a given dose of venom in salt solution readily undergo hemolysis in the presence of a non-lytic dose of horse serum. This activating action of horse serum Stephens demonstrated for the venoms of *Naja tripudians*, *Crotalus horridus*, *Daboia russellii*, and *Pseudechis porphyriacus*.

In 1902 Flexner and Noguchi² demonstrated the activating action of a group of sera and interpreted this action as due to serum complements similar to those involved in hemolysis by the complex serum lysins. Flexner and Noguchi were the first to conceive the venom hemotoxins as being of amboceptor structure.

Calmette,³ later, in 1902, found that certain sera heated at 62° C. showed an activating action for venom and that this property existed irrespective of the power of the serum to activate in the unheated state. From these findings Calmette concluded that the activation of venom could not be due to serum complements as held by Flexner and Noguchi, but that it was dependent rather upon the presence in the serum of a thermostabile "substance sensibilisatrice" (amboceptor). The ability of this substance to unite with the cell Calmette did not test. To explain those instances in which the serum possessed an activating action only after heating, Calmette assumed the presence in the normal serum of a thermolabile anti-hemolysin which masked the action of the thermostabile "substance sensibilisatrice," and whose destruction by heat removed the inhibition, leaving the "substance sensibilisatrice" free to effect hemolysis in conjunction with the venom.

¹ Thesis, University of Cambridge, November, 1898; also *Jour. Path. and Bact.*, 1900, 6, p. 273.

² *Jour. Exper. Med.*, 1902, 6, p. 277.

³ *Compt. rend. de l'Acad. Sci.*, 1902, 134, p. 1446.

In each of the three investigations cited, the fact was established beyond doubt that certain sera, modified or unmodified, exert a favorable influence on venom hemolysis. Concerning the manner in which such sera exert their influence, no consensus of opinion was reached. The explanation given by Calmette was in two points diametrically opposed to that given by Flexner and Noguchi: Whereas the latter workers considered the venom constituent to be the amboceptor, Calmette attributed this function to a thermostabile substance of the serum; and whereas Flexner and Noguchi contended that normal serum complements activate venom hemotoxin, Calmette denied an activation by such thermostabile substances. The findings as interpreted by these workers appeared mutually excluding.

Extending the work concerning serum activation I have been able to confirm the main findings given by the authors above cited but have found the complexity of serum activation to be far beyond explanation by any of the hypotheses given and indeed beyond explanation by any single hypothesis whatever. There is no general mode of activation which is common to all sera and therefore susceptible to one explanation.

The action of certain sera before and after heating shows clearly that the phenomena observed by Stephens and by Flexner and Noguchi are distinct from those discussed by Calmette. Ox serum, for example, when unheated shows an activation similar to that which Flexner and Noguchi referred to true complements and furthermore this activating action is entirely lost or reduced to a slight trace upon heating the serum as in the activation of true serum complements. But on the other hand if the serum be heated at a higher temperature there appears a second activating action of a different type and comparable to that observed by Calmette. This second activating power may be greater than that possessed by the unheated serum. The two types of activation as displayed by a single serum are shown in the following experiment (Table 39).

This experiment shows that in this instance there are two distinct activating actions, the one which is lost on heating the serum one-half hour at 56° C., and the second which appears on heating the serum at a higher temperature (65° C.). It was found, also, that the substance producing the second activation is far more stable than

indicated by Calmette in that it resists boiling for hours. Indeed the activating action of a serum is often greater when the serum is heated at 75° C. or boiled than when heated at 65° C.

TABLE 39.

AMOUNT OF OX SERUM $\frac{1}{10}$ C.C.	1 C.C. 5 PER CENT SUSPENSION HORSE ERYTHROCYTES			
	I Control Ox Serum Alone	II 0.02 C.C. 1 per cent Cobra Venom + Varying Amounts of Ox Serum:.		
		a) Normal	Heated $\frac{1}{2}$ hour at	
			b) 56° C.	c) 65° C.
0.5.....	faint trace	complete	faint trace	complete
0.35.....	o	almost complete	"	"
0.25.....	o	marked	o	"
0.15.....	o	slight	o	slight
0.1.....	o	trace	o	trace

The recognition of these two types of activation suggested the investigation of a considerable series of different sera. It was found that according to their activating action when fresh and when heated at various temperatures, the sera of the usual experimental animals fall into five groups. The first of these groups is composed of sera which show an activating action when fresh, when heated at 56° C., and also when heated at from 75° to 100°. Certain other sera which constitute the second group show no activating action either in the fresh state or when heated at 56° C., but only when heated at from 65° to 100°. Still other sera forming a third group show no activation either when fresh or heated at 56° but only on heating at the higher temperatures. The sera constituting the fourth group are those similar to the ox serum in the above experiment in that they activate when fresh, lack an activating power when heated at 56°, but show a second activation when heated at 65° or higher. The fifth group contains the single serum thus far observed which activates in the fresh state only, having no activating action either when heated at 56° or at the higher temperatures.

The combinations in which the above activations were observed are expressed in the following table (Table 40).

From this table it is seen that, with a single exception, all of the sera tested exhibit some grade of activation when heated between

75° and 100°, and furthermore that this activation is independent of the activating power of the same serum in the fresh state.

TABLE 40.

	ACTIVATING POWER OF SERUM			COMBINATIONS	
	a) Normal	b) Heated at		Serum	Erythrocytes
		56°	65°-100°		
I.....	+	+	+	horse horse horse man rabbit	ox goat* horse man ox
II.....	o	o	+	man man sheep rabbit	goat* ox sheep* goat*
III.....	o	+	+	ox sheep	ox ox
IV.....	+	o	+	guinea-pig ox guinea-pig	ox horse sheep*
V.....	+	o	o	guinea-pig	rabbit

* Slight hemolysis only, in these instances.

The many differences between the activating action of the several sera as observed in the fresh state, when heated at 56° C., at 65° C., and at 100° C., indicate the multiplicity of factors involved in serum activation considered as a whole. The insufficiency of Calmette's explanation is at once apparent on considering the sera of group I. Horse serum, for instance, shows as a rule the same activating power whether fresh or heated at 56°, whereas according to Calmette's assumption the destruction of the normal anti-hemolysin at 56° should greatly increase the action of the hypothetical thermostabile "substance sensibilisatrice." Also in the case of the sera contained in group II it is apparent that if the assumption were held that there exists a definite thermolabile anti-hemolysin destroyed at 56° C. it must also be assumed that in these particular sera the thermostabile "substance sensibilisatrice" of Calmette is lacking, inasmuch as these sera even when heated at 56° provoke no hemolysis in the presence of venom. Further heating of such sera, however, demonstrates that an activator is actually present. From the total results exhibited in the above table it appears that there is no single definite anti-hemoly-

sin in the sense of Calmette but that in the several sera there are inhibiting substances of varying thermostability whose action is more or less removed by heating the serum at different temperatures. The degree of heating required varies among the different sera and the amount of the activation afforded by the same degree of heat also varies between the several sera.

In view of the stability of the activator encountered in heated sera, the attempt was made to isolate an activating substance from normal serum by direct chemical procedure. To this end a quantity of serum was precipitated with 8–10 volumes of alcohol with the result that the activating substance of the serum was recovered in the alcoholic filtrate. Thus the alcoholic filtrate was evaporated *in vacuo* and the sediment so obtained suspended in a quantity of physiological salt solution equivalent to the original serum employed, with the result that the fluid was found to possess a marked activating action similar to that observed with the filtrate of heat-coagulated serum. Further isolation of the activating substance contained in such a suspension showed it to be lecithin—indeed it was in this connection that the activating action of lecithin was first discovered. The serum precipitate on the other hand when removed from the alcoholic filtrate and redissolved in water showed the distinct power of inhibiting the activating action of the filtrate and this inhibiting action of the precipitate was recognized as due to the serum proteins. Considering, then, the well recognized tendency of lecithin to form protein compounds it appears that in the native serum the lecithin is coupled with proteins, which coupling tends to limit the freedom of lecithin to react with the venom hemotoxin. In fact, in most unmodified sera it appears that no free lecithin exists, at least not in sufficient amount to activate the venom. When, however, factors are introduced, such as heat or alcohol coagulation, which sufficiently modify the proteins, the lecithin is freed from its combination and becomes available as an activator. The varying degrees of heat necessary with the several sera to liberate their lecithin content for activation shows that in the various sera, as in the various species of erythrocytes, the combinations of lecithin with the proteins differ widely. In selected instances, horse serum for example, the lecithin is often so lightly bound that it is at once available for the venom

reaction without the intervention of heat, while in other instances the opposite extreme is met, in that continued boiling barely suffices to liberate the lecithin from its protein compound. Between these extremes many intermediate compounds exist in the various sera, so that the degree of heating required to free the lecithin in a given serum is no criterion as to the degree necessary in a second instance. It seems probable moreover that in the same serum lecithin may be bound not to one protein only but may exist in several combinations of varying stability.

If the above conception of the relation of the proteins to the lecithin content of sera is held to account for the differences in lecithin activation displayed by different sera and by the same serum under different conditions, it might be expected that certain sera whose proteins unite firmly with lecithin would exhibit the power of taking up free lecithin and thus inhibiting its activating action. This is actually the case. Ox serum for example has been observed to completely inhibit the activating action of free lecithin, and in such instances the inhibiting power is fractionally removed by heating the serum at various degrees of temperature which are sufficient to modify and finally coagulate the proteins of the serum.

In view of these facts it appears that the heat modification of sera which effects an increase in their activating action for venom is not the destruction of a definite specific anti-hemolysin labile at 56° or even at 62°, but rather the breaking up at given temperatures of various protein compounds with the resulting liberation of the activating lipoid.

It cannot be maintained that of all lipoids liberated by heat modification of sera, lecithin is the only one which may modify venom hemolysis. It is true, however, that so far as observed, lecithin is the only such substance which effects a *true activation* of a venom hemotoxin. Inasmuch as the lack of recognition of a difference between the activating action of lecithin and that of other lipoids, including some of those of heated sera, is largely responsible for the confusions now existing in the literature concerning venom activation, this difference will be discussed in detail.

Used in its broadest sense the term activation has been employed up to this point to include all instances in which the addition of a

non-lytic dose of a third substance to venom and erythrocytes effects a hemolysis not otherwise occurring. Such a definition includes, therefore, the action of any substance which added to venom and non-susceptible corpuscles produces venom hemolysis. But the many substances which may be employed to this end are separable into two distinct classes according to the general mode of their action, viz.: those which react with the venom to elaborate a complete lysin, and secondly, those which act on the cell to modify its susceptibility.

The activation produced by the first group of substances is a true activation in the narrower sense, and is illustrated by the lecithin reaction with venom resulting in the formation of a lecithid as previously discussed.

In contrast to the true activation there is the activation of the second type which may be designated as indirect or pseudo-activation, in that the so-called activator does not actually enter into the formation of the complete lysin but only indirectly aids in the formation of that substance.

The rôle played by the pseudo-activators in this type of activation is that of rendering the intracellular lecithin of otherwise non-susceptible erythrocytes available for reaction with the venom hemotoxin and thus allowing the formation of the complex lysin—the lecithid.

The susceptibility of erythrocytes to a given venom is not absolute. Whenever the intracellular lecithin of erythrocytes is non-available for reaction with the venom hemotoxin, the erythrocytes are non-susceptible, and vice versa, when the intracellular lecithin is available for such a reaction, the erythrocytes are susceptible. Inasmuch as all erythrocytes contain an activating dose of lecithin, the factor determining their susceptibility is not the amount of lecithin contained, but rather the relation of this lecithin to the cell structure and any factor which may modify this relation may, therefore, modify the susceptibility of the cell to venom. A difference in the susceptibility of the same erythrocytes under differing conditions is illustrated by the following experiment in which the factor determining the susceptibility is nothing more than the menstruum in which the cells are suspended (Table 41).

TABLE 41.

AMOUNT OF COBRA VENOM 0.1 PER CENT C.C.	1 C.C. 5 PER CENT SUSPENSION OF SHEEP ERYTHROCYTES	
	a) In 0.85 per cent NaCl	b) In 10 per cent Cane Sugar
1.0.....	o	complete
0.75.....	o	"
0.5.....	o	"
0.35.....	o	"
0.25.....	o	"
0.15.....	o	"
0.1.....	o	marked
0.075.....	o	slight
0.05.....	o	almost o
0.035.....	o	o
0.025.....	o	o

From the above experiment it is seen that ox erythrocytes, which are never dissolved by cobra venom in physiological salt solution, are highly susceptible to that venom when suspended in a physiological sugar solution. In salt solution the lecithin of these cells is not available for reaction with the venom as it is when the same cells are suspended in sugar solution.

The susceptibility of erythrocytes may also be modified in the *same* menstruum. Those erythrocytes which are non-susceptible in physiological salt solution may be made susceptible in this menstruum by the addition of reagents which so modify the cell that the intracellular lecithin becomes available for a true activation of the venom hemotoxin. As indicated above the substances which act in this way constitute the class of indirect or pseudo-activators.

As early as 1903 in collaboration with Sachs¹ I pointed out the fact that the neutral fats, the fatty acids, and the soaps of the fatty acids all possess in some degree this power of favorably influencing venom hemolysis by modifying the availability of the intracellular lecithin. Repeatedly, however, the pseudo-activation produced by the lipoids has been confused with the true activation by lecithin.

As I previously pointed out, it is a significant fact that the lipoid pseudo-activators themselves are distinctly hemolytic. The hemolytic dose of these substances, moreover, bears a constant relation to the smaller dose required to effect the indirect activation. In other words, the pseudo-activation is produced by the addition of a sublytic dose of a lipoid hemolysin, and it is not surprising that a fraction of the dose which completely destroys the cell should suffice to some-

¹ Kyes and Sachs, *Berl. klin. Wchnschr.*, 1903, 40, pp. 21, 57, 82.

what modify its permeability or the equilibrium of the intracellular constituents.

The hemolytic action of olive oil and the relation of the lytic dose to the dose necessary for indirect activation is shown in Table 42.

TABLE 42.
HEMOLYSIS BY OLIVE OIL, WITH AND WITHOUT VENOM.

OLIVE OIL (SAT. METHYL ALCOHOL SOLUTION)* $\frac{1}{10}$ C.C.	1 C.C. 5 PER CENT SUSPENSION OX ERYTHROCYTES	
	a) Without Cobra Venom	b) +0.2 c.c. 1 per cent Cobra Venom
1.0.....	complete	complete
0.8.....	"	"
0.6.....	marked	"
0.4.....	o	"
0.2.....	o	"
0.1.....	o	"
0.08.....	o	"
0.06.....	o	o

* Throughout this and the following experiments with the fatty acids and the soaps the standard solution is a saturated methyl alcohol solution.

From this table it is seen that not only is this fat highly hemolytic but that the dose which is necessary to effect an indirect activation is not such a small fraction of a dose which causes complete destruction of the cell and a much smaller fraction of the dose which sufficiently damages the cell to allow some escape of hemoglobin.

The similar relation between the lytic and the indirect activating doses of a fatty acid is shown in the following experiment (Table 43) in which oleic acid is employed:

TABLE 43.
HEMOLYSIS BY OLEIC ACID, WITH AND WITHOUT VENOM.

OLEIC ACID (SAT. ALC. SOL.) $\frac{1}{100}$ C.C.	1 C.C. 5 PER CENT SUSPENSION OX ERYTHROCYTES	
	a) Without Venom	b) +0.2 c.c. 1 per cent Cobra Venom
1.0.....	complete	complete
0.5.....	"	"
0.25.....	trace	"
0.1.....	o	"
0.05.....	o	"
0.025.....	o	marked
0.015.....	o	o

This table shows that the amount of the fatty acid which is required to produce indirect activation with cobra venom is but one-tenth the amount which is required for complete destruction of the cells in

the absence of venom. The factor of the hemolytic dose and the activating dose is 1:10.

The action of the soap of the same fatty acid does not differ markedly from that of the acid itself as shown in the experiment given in Table 44.

TABLE 44.
HEMOLYSIS BY POTASSIUM SOAP OF OLEIC ACID, WITH AND WITHOUT VENOM.

POTASSIUM OLEATE (SAT. ALC. SOL.) 1/1000 C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES	
	a) Without Venom	b) +0.2 C.C. 0.1 per cent Cobra Venom
1.0.....	complete	complete
0.5.....	"	"
0.25.....	medium	"
0.1.....	o	"
0.05.....	o	marked
0.025.....	o	o
0.015.....	o	o

This table, when considered in conjunction with Table 43, shows that the factor in the case of the soap approximates that of the fatty acid and that there is here also a distinct relation between the lytic and the activating doses of the lipid.

For completeness the lytic dose, the activating dose, and the factor displayed by a series of fatty acids and their soaps are given in Table 45.

TABLE 45.

Doses Indicated in c.c. of a Saturated Methyl Alcohol Solution	Palmitic Acid	K Soap of Same	Isotrioxystearic Acid	K Soap of Same	Undecylinic Acid	K Soap of Same	Oleic Acid	K Soap of Same	Elaidic Acid	K Soap of Same	Capric Acid	K Soap of Same	Erucic Acid	K Soap of Same
Complete lytic dose without venom.....	0.1	0.2	0.1	..	0.05	0.07	0.005	0.0005	0.035	0.05	0.035	0.01	0.035	0.05
Complete lytic dose with co- bra venom....	0.015	0.025	0.015	0.1	0.0075	0.0025	0.0005	0.0001	0.0035	0.001	0.005	0.0025	0.005	0.0025
Factor (approx- imate).....	$\frac{1}{7}$	$\frac{1}{8}$	$\frac{1}{7}$..	$\frac{1}{7}$	$\frac{1}{4}$	$\frac{1}{10}$	$\frac{1}{6}$	$\frac{1}{10}$	$\frac{1}{60}$	$\frac{1}{4}$	$\frac{1}{4}$	$\frac{1}{7}$	$\frac{1}{20}$

An analysis of the above table shows that a certain variation exists between the factors displayed by the several lipoids, but throughout it is seen that some multiple of the activating dose completely destroys the cell.

Substances other than the lipoids, chloroform, for example, display somewhat the same action, and it is interesting to note that a sublytic dose of tetanus toxin has also been observed to produce an indirect activation of cobra hemotoxin. It would not be strange if ultimately non-lytic substances were found which may sufficiently modify the intracellular equilibrium to effect indirect activation without ever completely destroying the cell. Up to the present, however, I have observed no such substances apart from the various physiological solutions, such as the sugar solution previously considered in this section. With the exception of lecithin such lipoids as possess no hemolytic action show no indirect activating action. On the other hand, it is not of course maintained that a sublytic dose of each and all lysins can produce an indirect activation. The mode of action of the various lysins so far differs that the exact modification of the cell necessary for the liberation of the lecithin from its protein compound occurs only with a certain group of hemolysins. In generalizing then it can be said that a considerable number of lytic substances, when employed in sublytic doses, may so modify a non-susceptible cell as to render it susceptible to venom hemotoxin.

The indirect activation discussed above is entirely distinct from the true activation produced by lecithin. In this latter instance the activating action of the lecithin is in no way dependent upon the lytic action of lecithin as such upon the cell, but rather upon its power to react chemically with venom hemotoxin for the elaboration of the complete lysin—lecithid. In the case of the pseudo-activators, however, no similar end product is formed. Thus when cobra venom is added to a fat, a fatty acid, or the soap of the fatty acid, no chemical reaction occurs which results in the formation of a special lytic substance representing the lytic power of the venom. The mixture of any of these lipoids with cobra venom fails, even on standing, to produce a modification of the rate of hemolysis as compared with that observed when these substances are added coincidently with the blood. There is, in other words, no obliteration of the incubation period such as must result were the action of these lipoids comparable to the true activation produced by lecithin. The degree of heat, moreover, required for the destruction of cobra venom in the presence

of the above lipoids is the same as that required in the absence of these substances, indicating that there is no formation of a thermostabile hemotoxin-lipoid compound, comparable to the lecithid.

In view of these facts concerning the action of the pseudo-activators there seems to be no necessity for further confusion between the indirect action of the lipoids in general with the true activation produced by lecithin.

Now, in the case of heated sera certain lipoids doubtless play a rôle as indirect activators in selected instances; true activation when produced by such sera however is invariably due to their lecithin content. Moreover, the conditions under which a given serum is heated may determine whether the activation which it produces be the indirect activation of the lipoids in general or the true activation by lecithin. Among such conditions are: the degree of heat, the dilution of the serum as heated, the degree of alkalinity of the serum, the freshness of the serum, the amount of free hemoglobin contained, the salt content, etc.

Up to this point the consideration of the activating action of sera has been confined to that of heated sera and it has been shown that the heating which increases the activating action does not produce this change by destroying an anti-hemolysin in the sense of Calmette, but rather by liberating from their protein compounds certain normal lipid constituents of the serum. Furthermore, it has been shown that the activating action of these free lipoids is of two distinct types, that of all but lecithin being effected by a modification of the cells themselves, whereas that effected by lecithin is a true activation of the venom hemotoxin.

It now becomes of importance to discuss the activating action of fresh unheated sera, and the question at once arises in this connection as to whether or not the activating action of fresh sera may be attributed to the same substances which operate in the case of the heated sera.

Undoubtedly there is a small number of fresh sera whose activation is similar to that produced by the same sera when heated. Such sera are those included in group I, namely, those whose activating action is but slightly, if at all, modified by heating. In a greater number of instances, however, the activation displayed by fresh serum

is susceptible of differentiation from that of heated serum in that the activating substance appears to be thermolabile. An example of such a serum is seen in each of the two following experiments, in which the activating action displayed by the fresh serum is lost on heating at 56° C. for one-half an hour (Tables 46 and 47).

TABLE 46.

AMOUNT OF GUINEA-PIG SERUM $\frac{1}{3}$ C.C.	1 C.C. 5 PER CENT SUSPENSION OF SHEEP ERYTHROCYTES		
	a) Normal Serum with- out Venom	b) 0.02 c.c. 1 per cent Cobra Venom +	
		1) Guinea-Pig Serum, Normal	2) Guinea-Pig Serum Heated $\frac{1}{2}$ Hour at 56° C.
0.5.....	slight	complete	o
0.25.....	trace	marked	o
0.1.....	o	slight	o
0.05.....	o	trace	o
0.025.....	o	faint trace	o
0.01.....	o	o	o

TABLE 47.

AMOUNT OF OX SERUM $\frac{1}{16}$ C.C.	1 C.C. 5 PER CENT SUSPENSION OF HORSE ERYTHROCYTES		
	a) Normal Serum with- out Venom	b) 0.02 c.c. 1 per cent Cobra Venom +	
		1) Ox Serum, Normal	2) Ox Serum Heated $\frac{1}{2}$ Hour at 56° C.
0.5.....	faint trace	complete	faint trace
0.35.....	o	almost complete	" "
0.25.....	o	marked	o
0.15.....	o	slight	o
0.1.....	o	trace	o

From the above experiments it is seen that, whereas the fresh serum both of guinea-pig and of ox displays a certain activating power for cobra venom, the same serum when heated at 56° loses this activating action. Similar results have been observed also in the following combinations:

Horse corpuscles.....	Ox serum
Ox "	Guinea-pig serum
Sheep "	" "
Rabbit "	" "

This possession by fresh sera of an activating power, which disappears on heating at the temperature employed for the inactivation of serum hemolysins in general, would seem to indicate that the

activation produced by these sera is dependent upon true serum complements rather than upon thermostabile activators similar to those discussed above for heated sera.

It must be recognized, however, that the indications afforded by such experiments do not alone constitute absolute proof that even here lecithin may not be the activating substance.¹ There exist in addition, however, certain indications, which taken in conjunction with those of the above experiments seem to show rather conclusively that the activating action of the fresh sera under discussion is not a lecithin activation. These indications are observed in differences which appear in the hemolysis produced by venom when activated by the fresh sera and by lecithin. Thus the rate of hemolysis produced by the serum complements is much less rapid than that occurring with lecithin. In lecithin activation with fairly large doses of cobra venom the hemolysis is immediate, while in the case of the serum activation a considerable incubation period is constant. In the case of lecithin activation, also, the hemolysis proceeds at 0° C. while the activation by the sera under discussion occurs only at a higher temperature.

An additional point which seemingly differentiates the activation by guinea-pig serum for instance from that by lecithin is the difference in the susceptibility of these activators to the inhibiting action of cholesterin. The lecithin activation is markedly inhibited by traces of this substance, whereas the serum activation is but relatively slightly influenced. Table 48 indicates this difference in detail.

This table shows that an amount of cholesterin one hundred times greater than that required to completely inhibit lecithin activation is insufficient to completely inhibit the hemolysis with guinea-pig serum activation.

A somewhat similar differentiation of the guinea-pig serum activation and that of lecithin is furnished also by the following experi-

¹ It might be conceived, for instance, that in such fresh sera, where the lecithin is so lightly bound to the protein that it is free to activate venom, the proper heat modification of the serum might so change the protein lecithin compound as to render the lecithin unavailable for the venom reaction. In fact, in an earlier section of this paper it has been shown that lecithin heated in a solution of hemoglobin is actually so bound to that substance as to lose its activating action, and it was further shown that hemoglobin when heated alone in an aqueous solution acquires the power of inhibiting the activating action of lecithin subsequently added. In view of these facts the simple removal of the activating action of the serum for cobra venom by heat inactivation at 56° C. does not constitute absolute proof that the activating substance itself is thermolabile and hence not lecithin. Indeed a broader statement seems justified, viz., that heat destruction of the activating action of sera in general does not constitute absolute proof that the activating substance itself is thermolabile.

ment, in which an artificial serum, so prepared as to lack an inhibiting action for lecithin activation, still produced a distinct inhibition of the serum activation. The alcohol precipitate of rabbit serum was redissolved in salt solution and the anti-lecithin action of this solution was eliminated by the addition of an excess of lecithin. In other

TABLE 48.
CHOLESTERIN INHIBITION.

CHOLESTERIN SOLUTION*	1 C.C. 5 PER CENT OX ERYTHROCYTES+0.01 C.C. 1 PER CENT COBRA VENOM+ACTIVATING DOSE OF:	
	a) Guinea-Pig Serum	b) Lecithin
0.5.....	medium	o
0.25.....	"	o
0.1.....	"	o
0.05.....	marked	o
0.025.....	complete	o
0.01.....	"	o
0.005.....	"	o
0.0025.....	"	complete

* Cholesterin solution made by adding 1 c.c. of a hot saturated methyl alcohol solution of cholesterin to 9 c.c. of 0.85 per cent salt solution.

words, the serum thus prepared, far from possessing an anti-lecithin action, contained sufficient free lecithin to produce a lecithin activation, when employed in sufficient amount. This serum, however, possessed the same power of inhibiting guinea-pig serum activation as did a portion of the similarly prepared serum, to which however no lecithin was added. The details of this experiment with the tabulated results are given below (Table 49).

Twenty c.c. of rabbit serum were precipitated with 180 c.c. of absolute alcohol, the precipitate recovered quickly, and redissolved in 20 c.c. of salt solution. This solution

TABLE 49.

AMOUNT OF INHIBIT- ING SOLUTION C.C.	HEMOLYSIS ALLOWED BY	
	a) Inhibiting Solution without Lecithin*	b) Inhibiting Solution + Lecithin*
0.1.....	faint trace	complete
0.5.....	trace	almost complete
0.25.....	slight	medium
0.15.....	"	slight
0.1.....	medium	medium
0.05.....	"	"
0.025.....	marked	marked
0.01.....	"	almost complete
0.....	complete	complete

* 0.25 c.c. of guinea-pig serum and the inhibiting solution were allowed to stand three-quarters of an hour in contact at 37° C. prior to the addition of 0.01 c.c. 1 per cent cobra venom and 1 c.c. 5 per cent suspension of ox erythrocytes.

showed an inhibition of venom hemolysis both by lecithin activation and by guinea-pig serum activation.

Four c.c. of the inhibiting solution were then allowed to stand three-fourths of an hour with 2 c.c. of a 0.17 per cent lecithin solution. This mixture was then seen to possess a sufficient excess of free lecithin to activate cobra venom when sufficiently large doses of the former were employed. Smaller doses on the other hand sufficed to inhibit the activation by guinea-pig serum to the same degree as the original solution without adding lecithin.

In the above experiment it is seen that an artificial serum, which possessed no anti-lecithin action, still produced distinct inhibition of the fresh serum activation.

A further indication that the activation by the fresh sera under discussion is not due to their lecithin content is the fact that such sera possess the power of actually inhibiting the action of free lecithin. Of the same guinea-pig serum which produces activation in optimum doses, smaller doses suffice to inhibit totally the activating action of a dose of free lecithin. Such a result is seen in the following experiment (Table 50):

TABLE 50.
INHIBITION OF LECITHIN ACTIVATION BY ACTIVE GUINEA-PIG SERUM.

Amount of Guinea-Pig Serum c.c.	1 c.c. 5 per cent Suspension of Ox Erythrocytes + 0.001 c.c. 1 per cent Cobra Venom + 0.075 c.c. 0.025 per cent Lecithin*
0.5.....	complete
0.25.....	marked
0.1.....	trace
0.05.....	o
0.025.....	o
0.01.....	trace
0.....	complete

* Lecithin and serum allowed to stand one-half hour at 37° C. prior to addition of venom and erythrocytes.

It is scarcely conceivable that the same serum which inhibits lecithin activation should produce a lecithin activation.

The fact that papain digestion of the sera under discussion destroys their activating action and on the other hand does not modify the activating of lecithin is an additional point which indicates that it is not the lecithin content of these sera which gives them their activating power. Digestion of 5 c.c. of guinea-pig serum with 1 c.c. of 10 per cent papain solution for one and one-half hours according to the procedure introduced by Ehrlich and Sachs, resulted in the

complete destruction of the activating action of the serum. Table 51 shows the activating action of the same guinea-pig serum before and after such papain digestion.

TABLE 51.

AMOUNT OF SERUM C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.02 C.C. 1 PER CENT COBRA VENOM + GUINEA-PIG SERUM	
	a) Normal	b) Digested with Papain
0.5.....	complete	almost 0
0.35.....	"	" 0
0.25.....	almost complete	" 0
0.15.....	marked	" 0
0.1.....	"	0
0.075.....	medium	0

TABLE 52.

AMOUNT OF LECITHIN SOLUTION C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.02 C.C. 1 PER CENT COBRA VENOM + 0.025 PER CENT LECITHIN SOLUTION	
	a) Untreated	b) Digested with Papain
0.25.....	complete	complete
0.15.....	"	"
0.1.....	"	"
0.075.....	trace	trace
0.05.....	0	0

From Table 51 it is seen that the activating action of the guinea-pig serum was destroyed by papain digestion whereas Table 52 shows that lecithin when subjected to the same treatment retains its full activating power. Similar results are obtained also in parallel experiments in which treatment with HCl and with NaOH is substituted for the papain digestion.

Although the proof is not to be considered absolute, such findings as those referred to above indicate strikingly the probability that the activation by such sera as are inactivated at 56° C. is not due to their lecithin content.

The question now arises as to the manner in which these sera do effect venom activation and at the outset it should be stated that no consensus of opinion has yet been reached which justifies a definite answer to this question. Assuming for the present, however, that the hemotoxin which is activated by the serum complement is the same as that activated by lecithin, it is at once apparent that the activating action of the fresh serum may be either direct or indirect; in

other words, that the thermolabile activating substances react with the hemotoxin itself or that they act upon the cell to liberate endocomplements. Although this question, as to whether the activating action of fresh sera is a direct or indirect activation, is not definitely answered by such data as are now available, the weight of evidence appears to me to favor the view that the activation is indirect.

Flexner and Noguchi,¹ in their early work concerning serum activation, took the position that a given venom contains "several or many intermediary bodies" and that "these bodies show specific affinities for certain (serum) complements" (p. 288). According to this scheme it was assumed that given corpuscles bound certain venom amboceptors and that these amboceptors were then susceptible to activation by specific serum complements. In other words, a true activation of the venom hemotoxin by serum complements was assumed to occur after the manner of the complement-amboceptor reaction of the true serum hemolysins.

The experiments of Flexner and Noguchi, which at first appeared convincing, do not however, in the light of subsequent work, justify the closeness of the analogy which these authors drew between the activating action of sera in the case of the complex serum lysins and in the case of the venom hemotoxins. It is not meant to argue that venom hemotoxins are no longer to be considered as amboceptors, but rather to show that the early experiments given as proof of a true activation of venom hemotoxin by thermolabile serum complements are inconclusive and in part unreliable. The findings of Kyes, Lamb, and Sachs all show that there is no selective binding of venom amboceptors by the various species of washed erythrocytes which justifies the assumption of "several or many intermediary bodies"—the binding power of the erythrocytes is far too slight to allow a differential determination of multiple hemotoxins in a given venom as attempted by Flexner and Noguchi. As regards the experiments which Flexner and Noguchi advanced to show a true activation of venom amboceptors by serum complements, it is to be remembered that throughout these experiments Flexner and Noguchi proceeded under the false impression that venoms alone lack the power of dissolving erythrocytes and that, as a result, the

¹ *Jour. Exper. Med.*, 1902, 6, p. 277.

bulk of the experiments were performed with erythrocytes which are themselves susceptible to venom alone. The controls given in these instances are insufficient to justify any conclusion as to the rôle of the sera employed.¹ In view of these facts the work of Flexner and Noguchi is insufficient as proof either of a multiplicity of hemolytic amboceptors in a given venom or of a reaction between the thermolabile complements of sera and venom hemotoxin analogous to that observed with the complement-amboceptor complex of the serum hemolysins.

More recently and from a point of view differing somewhat from that of Flexner and Noguchi, von Dungern and Coca² also have attempted to demonstrate a multiplicity of cobra venom hemotoxins and a true serum complement activation of this venom. These authors maintained that there are two distinct types of hemotoxin in cobra venom and that one of these is activated by serum complements whereas the other is not. The hemotoxin which von Dungern and Coca conceived as being activated by serum complements they considered as a true amboceptor readily bound to the erythrocytes, whereas the other hemotoxin which is activated only by lecithin they refused to accredit as an amboceptor in any sense of the term.

Von Dungern and Coca treated non-susceptible erythrocytes (ox) with a strong venom (cobra) solution and after removal of the venom solution determined the hemolysis occurring upon the addition of guinea-pig serum and upon the addition of lecithin to the treated corpuscles. They found that the addition of guinea-pig serum resulted in hemolysis, whereas the addition of lecithin produced no hemolysis. Then testing the venom solution with which the corpuscles had been treated, for its power to dissolve normal erythrocytes both in the presence of lecithin and of guinea-pig serum, they found that hemolysis resulted in both instances. It was from these findings that von Dungern and Coca made their deduction as to the presence of multiple hemotoxins in cobra venom and the true serum activation of one of these.

Briefly stated, the results given by von Dungern and Coca are inconstant and when actually occurring indicate conclusions quite

¹ Cf. experiments, *op. cit.*, p. 288.

² *Münch. med. Wchnschr.*, 1907, 4, p. 2317.

the opposite of those cited. Neither the tests with the erythrocytes nor those with the supernatant venom solution demonstrate the specific binding of an amboceptor or the true activation of such an amboceptor by a serum complement. The simple fact that the supernatant venom from which the amboceptor was supposedly removed by the corpuscle showed hemolysis when tested with fresh corpuscles and guinea-pig serum indicates at once that von Dungern and Coca did not, as they claimed, effect the complete separation of two distinct hemotoxins. The venom solution in question retained after treatment with the corpuscles its power to effect hemolysis not only with lecithin but with the serum as well. It is significant that just in this connection von Dungern and Coca omitted the quantitative details in their experiments and casually referred the hemolysis produced with guinea-pig serum to the activating action of the lecithin content of the serum. Fortunately however Sachs¹ has furnished details in this regard and in repeating von Dungern and Coca's experiments showed that the hemolytic power of the venom with guinea-pig serum is practically the same subsequently to the treatment of the venom with ox corpuscles as prior.

In view of the fact that von Dungern and Coca found the venom which they removed from the ox corpuscles in their experiment still to contain a hemotoxin susceptible to activation by guinea-pig serum and further in view of the fact, as shown by Sachs, that the amount of such hemotoxin is practically the same as that in the original venom solution, the claim by von Dungern and Coca that they actually accomplished a complete separation of two hemotoxins in cobra venom is without force. In fact, influenced by the results of Sachs and by a repetition of their own experiments von Dungern and Coca have themselves more recently relinquished their claims both as to a multiplicity of hemotoxins in cobra venom and as to a true activation by serum complements.²

A consideration then of the data thus far accumulated fairly allows the statement that there exist at present no proofs that serum complements other than lecithin react with the venom hemotoxin to effect a true activation.

¹ *Münch. med. Wchnschr.*, 1908, 55, p. 437.

² *Biochem. Ztschr.*, 1908, 9, p. 407.

The above statement is far from saying, however, that active sera have no influence on venom hemolysis or that non-susceptible corpuscles are indifferent to treatment with concentrated venom solution. Early in my work with the venoms I found that non-susceptible erythrocytes when treated with concentrated cobra venom were more readily dissolved by complement-containing sera than were unheated corpuscles and it was this finding which led me to accept for a time the opinion held by Flexner and Noguchi that a true activation of the venom amboceptors was effected by serum complements. Later in the work, however, when recognizing more fully the intracellular activating action of lecithin and the extensive rôle played by lytic substances as indirect activators, it appeared to me that the phenomena observed with active sera did not require the interpretation given by Flexner and Noguchi, but that an indirect action of the serum favoring intracellular lecithin activation was more probable. At present I am more convinced that such is the case.

Earlier in the present section I have shown that a considerable number of lytic substances, when employed in sublytic doses and in conjunction with cobra venom, effect a hemolysis not otherwise occurring. This indirect activation has been referred, moreover, to a modification of the erythrocytes rendering the intracellular lecithin available for reaction with the venom hemotoxin.

Now it may be stated that with but a few, if indeed with a single exception, those sera which favorably influence venom hemolysis when fresh and which lose this power when inactivated, possess in some degree a lytic action of their own for the corpuscles tested. Moreover, the degree of the influence of such active sera upon venom hemolysis is in general directly proportionate to the hemolytic power of the serum itself in the absence of venom. Thus the serum most extensively used for the demonstration of an activation is that of the guinea-pig, employed in conjunction with ox corpuscles, and this serum invariably shows a distinct lytic action of its own for those corpuscles (Table 53).

It will be observed in Table 53 that whereas 0.5 c.c. of the serum was required to effect complete hemolysis in conjunction with the venom, the same amount even without venom produced a trace of

hemolysis. The factor between the lytic and the activating doses cannot be given in the above experiment on the basis of the complete lytic dose but it is to be noted that the factor indicated by the maximum non-lytic dose is well within the range of those factors observed with the lipoid indirect activators. Thus whereas 0.1 c.c. was the maximum non-lytic dose with the venom, amounts of serum above 0.25 c.c. sufficed to effect some grade of hemolysis in the absence of venom. Based upon the "nil" dose the factor in this instance is $1/2.5$. Comparable results are constantly observed in all cases where the above combination is employed. (Cf. Kyes, Sachs, and von Dungern and Coca.)

TABLE 53.
GUINEA-PIG SERUM ACTIVATION.

GUINEA-PIG SERUM c.c.	1 C.C. 5 PER CENT SUSPENSION OX ERYTHROCYTES	
	a) Without Venom	b) +0.2 c.c. 0.1 per cent Cobra Venom
1.0.....	slight+	complete
0.75.....	slight-	"
0.5.....	trace	"
0.35.....	faint trace	almost complete
0.25.....	o	marked
0.15.....	o	trace
0.1.....	o	o
0.075.....	o	o

Likewise in the combination of other sera with other corpuscles a similar lytic action is to be observed. Thus in experiments showing the influence of ox serum on sublytic doses of cobra venom for horse corpuscles a lytic action of the serum for these corpuscles is seen (Table 54).

TABLE 54.

OX SERUM c.c.	1 C.C. 5 PER CENT SUSPENSION OF HORSE ERYTHROCYTES	
	a) Without Venom	b) +Sublytic Dose of Cobra Venom
1.0.....	complete	complete
0.5.....	"	"
0.25.....	almost complete	"
0.1.....	slight	"
0.05.....	faint trace	"
0.025.....	o	marked
0.01.....	o	o

In the above experiment, as in the previous one, it is seen that the amount of serum required to effect complete hemolysis with the venom

not only modified the cell in the absence of the venom, but sufficiently so as to allow the escape of hemoglobin.

Further striking indications of the dependence of the activating action of the serum upon its lytic action are also to be found in the work of von Dungern and Coca. Although the significance of their results was overlooked by these authors, the protocols show that three of the four rabbit sera which they tested and which produced no activation of cobra venom showed also no lytic action of their own in the absence of venom. On the other hand, a fourth rabbit's serum which these authors found to possess an activating power for the venom is seen to possess a hemolytic action of its own. In other words, the sera which showed no lytic action in the absence of the venom showed no activation in the presence of the venom. And, vice versa, the serum which showed a lytic power of its own did produce an apparent activation in the presence of venom.

Now in the absence of actual proof as to a true activation of venom hemotoxin by thermolabile complements and in view of the fact that lytic substances in sublytic doses are known to modify the availability of intracellular lecithin for venom, the above considerations strongly indicate, at least, that the activation of venom by the active sera under discussion is a pseudo-activation, in which the complex serum lysins (complement-amboceptor complex) act simply after the manner of the lipoid lysins in that they indirectly facilitate a reaction between the intracellular lecithin and the venom hemotoxin.

The above explanation accounts, however, only for those instances in which the serum, the venom, and the corpuscles are coincidentally present. It is not applicable to those instances where corpuscles treated with venom show an increased susceptibility to active sera. Although the results are not constant, it is nevertheless true that certain non-susceptible corpuscles when treated with concentrated venom in salt solution and when removed from such a solution may be dissolved by an amount of serum which was non-hemolytic for the same corpuscles prior to such treatment. Even here, however, it is not necessary to assume, as was first done, that there is a binding of a venom constituent to the corpuscles and that this constituent is then activated by a serum complement.

It cannot rightly be assumed that those corpuscles which show no

hemolytic reaction with the venom in salt solution therefore suffer no structural modification. It is rather to be remembered in this connection that the venom is a composite of toxic principles, of which the hemotoxin is but one, and further that these same cells when suspended in a physiological sugar solution instead of the salt solution are completely destroyed by the venom. From this point of view it does not appear improbable that the treatment of the corpuscles with the strong venom in salt solution, although not damaging the cell to the extent of allowing hemolysis, does, however, produce profound structural modifications and that these destructive changes so decrease the resistance of the corpuscles as to allow their complete destruction by smaller doses of the complex serum lysins than required in the case of the untreated corpuscles. This explanation of the phenomena observed, although not established beyond doubt, is, however, so far in accord with the data thus far accumulated as to eliminate the necessity of assuming the activation of venom amboceptors bound to the corpuscles by true serum complements.

The exact modifications of cells which may occur in the above instance, as in the case of the indirect activators, are not known. Doubtless many substances will eventually be recognized which may modify the cell susceptibility by rendering its membrane more permeable, by extracting intracellular inhibiting substances, by modifying the arrangement of intracellular substances, or possibly by acting as catalyzers in the reaction of the lysin with the cellular constituent upon which it operates. In all attempts to analyze such modifying conditions, however, it is necessary to recognize distinctly the difference between the action of intracellular substances in accelerating the rate of hemolysis and in increasing the degree of hemolysis. The addition of certain constituents of serum and indeed of corpuscles themselves to a lysin and given corpuscles may markedly increase the rate of hemolysis without influencing however the degree of hemolysis ultimately to be observed. I emphasize the necessity of this distinction, inasmuch as it has been repeatedly overlooked by recent workers. Such readings, for instance, as those made after one and one-half, two, and four hours by von Dungern and Coca throughout their experiments are of but little value as quantitative data concerning the amount of hemotoxin actually present in a given instance. Such observations are of

value only as indicating the rate of hemolysis. The end reaction, which in most cases of venom hemolysis can be observed only from ten to twenty-four hours, is the only safe criterion as to the true hemolytic power of a given toxin. By the way of illustration the following experiment serves to show the influence of an aqueous extract of guinea-pig erythrocytes in strikingly increasing the rate of venom hemolysis without modifying the extent of such hemolysis (Table 55).

TABLE 55.

To each tube of one series (I) was added 1 c.c. of physiological salt solution and to each tube of the second series (II) was added 1 c.c. of the salt solution employed for the second washing of the corpuscles and representing aqueous extract of same.

AMOUNT OF COBRA VENOM (0.02 PER CENT) C.C.	1 C.C. 5 PER CENT SUSPENSION OF GUINEA-PIG ERYTHROCYTES HEMOLYSIS OBSERVED AFTER:			
	A. $\frac{1}{2}$ Hour at 37° C.		B. 2 Hours at 37° C. and 16 Sub- sequent Hours at 10° C.	
	I) +1 c.c. Salt Solution	II) +1 c.c. Wash Water	I) +1 c.c. Salt Solution	II) +1 c.c. Wash Water
1.0.....	o	complete	complete	complete
0.75.....	o	"	"	"
0.5.....	o	"	"	"
0.35.....	o	"	"	"
0.25.....	o	"	complete	complete
0.15.....	o	marked	almost complete	almost complete
0.1.....	o	medium	medium	marked
0.075.....	o	faint trace	slight	slight
0.05.....	o	o (?)	almost o	faint trace
0.035.....	o	o	o	o
0.025.....	o	o	o	o
0.015.....	o	o	o	o

In the above experiment it is seen that conditions which may have no effect upon the ultimate hemolysis produced by cobra venom may nevertheless markedly increase the rate of hemolysis, and that any reading of the comparative degree of hemolysis prior to the end reaction will lead to a fallacy concerning the hemolytic power of the same venom under the two conditions.

In concluding this section concerning serum activation I wish to repeat the statement made earlier in the discussion that the data thus far obtained justify only provisional conclusions, and, further, to emphasize the fact, that although sera are known to modify venom hemolysis in several, if not many ways, there exists at present no proof of a true activation of venom hemotoxin by serum constituents other than lecithin.

VI.

GENERAL CONSIDERATIONS.

In the present section it is intended to discuss certain more or less isolated phases of venom hemolysis which do not fall logically under the headings of the previous sections.

The discovery of the activating action of lecithin for the venoms stimulated a wide appreciation of the important rôle of the lipoids in the field of immunity. Among other results, it has occurred that numerous investigators have attempted to effect, under dissimilar conditions, activations similar to that displayed by lecithin with the venoms. The lecithin reaction appears, however, to be one of not very general occurrence and unfortunately much confusion has arisen from attempts to draw close analogies where such analogies do not exist. Thus the results obtained by Pascucci¹ with lecithin and ricin, by Landsteiner and Jagic² with lecithin and silicic acid, by Reiss³ with chloroform solutions of lecithin and trypsin ferment, by Iscovesco⁴ with lecithin and colloidal iron; all, while adding excellent data concerning certain properties of lecithin, are not to be confused as examples of a true lecithin activation similar to that observed in the formation of venom lecithids. In these instances, no evidence of a chemical reaction is furnished by the isolation of a newly formed end product and indeed there is no indication whatsoever of a chemical reaction between the substances involved. The phenomena observed are referable in the individual instances either to a physical mixture of the reagents or to an independent action of the two reagents upon the cell indicator employed.

Also Michaelis and Rona⁵ have failed to draw a distinction between certain physical mixtures and the chemical reaction which occurs in the formations of venom lecithids. These authors found that rennin is precipitated from its aqueous solution by mastix and that the precipitate thus obtained is soluble in chloroform. On the basis of this finding and the correspondence in chloroform solubility of mastix-rennin with that of the cobra lecithid, these authors have attempted

¹ *Beiträge z. chemisch. Phys. u. Path.*, 1906, 7, p. 457.

² *Wien. klin. Wchnschr.*, 1904, 17, p. 63.

³ *Berl. klin. Wchnschr.*, 1904, 45, p. 1169.

⁴ *Compt. rend. de la Soc. de Biol.*, 1907, 63, p. 744.

⁵ *Biochem. Ztschr.*, 1907, 4, p. 11.

to establish a complete analogy between the mastix-*rennin* precipitation and the lecithin-venom reaction, reaching the conclusion that in the latter instance, as in the former, the essential reaction is that of a physical absorption by two colloids.

Now in section IV it has been shown that the end product of the reaction between lecithin and venom is, so far as can be determined by an elementary chemical analysis, composed largely of monostearyl-lecithin. It is at once necessary, therefore, in making any possible application of the hypothesis advanced by Michaelis and Rona, to assume that at first there actually does occur a chemical reaction in the splitting of monostearyl-lecithin from lecithin, and that secondly there occurs an absorption of the active hemotoxin constituent by this split product. The final question determining the validity of the Michaelis-Rona hypothesis would then be, whether or not the venom lecithid is a mixture of a split product of lecithin and native hemotoxin or is a single newly formed hemolysin.

The physical and chemical proofs that cobra lecithid is indeed a newly formed substance have been discussed in section IV but I may again point out in this connection the fact that the lecithid as a toxin displays distinctive characteristics which show that its lytic action cannot be referred to either of the substances employed in its production, as such. Among these characteristics are the following :

1. The extreme rapidity of its hemolytic action;
2. The absolute heat resistance, even to boiling;
3. The lack of susceptibility to the neutralizing action of antivenin, and
4. The possibility of producing with the lecithid a new antibody of greater affinity than that possessed by antivenin.

Furthermore, it is not possible to recover from the lecithid a trace of lecithin or of native hemotoxin; and again as shown in the following table, the hemolytic action of the lecithid is not increased either by the addition of unmodified venom or of free lecithin (Table 56).

Contrasting sharply with the characteristics of the lecithid which show it to be a newly formed chemical substance, are the properties of the mastix-*rennin* preparations of Michaelis and Rona. The details given by these authors show that unmodified *rennin* may be recovered from their preparations: Also the *rennin* as mixed with the mastix is seen to retain marked characteristics of native *rennin* among which is an identical susceptibility to destruction by heat.

The mastix-rennin preparations described by Michaelis and Rona do indeed give all evidence of being physical mixtures and such only. In this however they differ so essentially from cobra lecithid that analogies between these two classes of preparations are unwarranted and deductions therefrom unreliable.

TABLE 56.
INDIFFERENCE OF COBRA LECITHID TO PRESENCE OF LECITHIN OR OF NATIVE VENOM.

COBRA LECITHID 0.1 PER CENT c.c.	1 C.C. 5 PER CENT SUSPENSION OF GOAT ERYTHROCYTES		
	a) Cobra Lecithid Only	b) Cobra Lecithid + 0.1 c.c. 0.1 per cent Lecithin	c) Cobra Lecithid + 0.2 c.c. 0.1 per cent Unmodified Cobra Venom
1.0.....	complete	complete	complete
0.75.....	"	"	"
0.5.....	"	"	"
0.35.....	"	"	"
0.25.....	almost complete	almost complete	almost complete
0.15.....	medium	medium	medium
0.1.....	almost 0	almost 0	almost 0
0.075.....	0	0	0
0.05.....	0	0	0
0.035.....	0	0	0
0.025.....	0	0	0
0.015.....	0	0	0

In contrast to the contributions above cited in which the criteria of true lecithin activation have, it appears to me, been more or less confused stands the work of Morgenroth and Carpi¹ concerning a lecithin activation of bee poison. These authors have demonstrated a true lecithin activation of bee-poison hemotoxin by isolating a lecithid of that constituent and their results are conclusive in showing that the hemotoxin of this secretion is comparable in its action to the hemotoxins of the snake and scorpion venoms. Also this work corroborates the chemical nature of the reaction between native hemotoxins and lecithin in the elaboration of lecithids. The work of Friedemann² concerning lecithin activation of a hemolytic principle extracted from the pancreas displays certain indications of a true activation. Unfortunately, however, Friedemann has not as yet produced final proof by isolating a lecithid and his results are, therefore, less conclusive than those of Morgenroth and Carpi.

Confusion arising from forced analogies is also to be observed in certain phases of the work of Noguchi on venom hemolysis. In

¹ *Berl. klin. Wchnschr.*, 1906, 43, p. 1424.

² *Deut. med. Wchnschr.*, 1907, 33, p. 585.

a given instance for example,¹ this investigator, while admitting the activating action of lecithin in general, has sought to show that the intracellular lecithin of erythrocytes is not available for venom activation, and that the true endocomplement of susceptible corpuscles is a fatty acid.

In a previous section I have emphasized the distinction long since made, between the true activation by lecithin (including cephalin) and the pseudo-activation by the fatty acids, etc., pointing out that in the latter instance the rôle of the fatty acids is not that of reacting with the venom, but of rendering the intracellular lecithin available for its reaction with the venom. It is in confusing this indirect action of the fatty acids with true activation, that Noguchi has been led to assume that these substances are essential intracellular activators which unite with the venom to form the ultimate lysin.²

The fundamental difficulty in accepting an interpretation such as that advanced by Noguchi is that all proof is lacking to show that a single one of the fatty acids or its soap, when isolated, is capable under any conditions of effecting a true activation of a venom. Before serious consideration can be given any substance as the essential intracellular venom activator it must first be shown that this substance in some form actually possesses the power of reacting with the venom to form a hemolytic substance. This has not been shown in the case of the fatty acids and their soaps and indeed Noguchi himself in a more recent publication ceases to draw strict analogies between the action of these substances and the activation by lecithin.

The fact that it is possible to extract with ether certain fatty acids from susceptible corpuscles which are not likewise obtainable from non-susceptible corpuscles is in full accord with the conception of intracellular lecithin activation: In those corpuscles which are susceptible to venom by virtue of the relatively loose combination of lecithin with their stromata, it might well be expected that other lipoids present were also loosely bound and hence relatively susceptible to extraction. It may indeed be possible that the presence of certain fatty acids in the stromata may be among the factors which determine the availability of the intracellular lecithin for reaction with

¹ *Jour. Exper. Med.*, 1907, 9, p. 436.

² Arrhenius (*Biochem. Ztschr.*, 1908, 11, p. 161) also fails to make a sufficient distinction between the action of lecithin with cobra venom and that of sodium oleate.

the venom. It cannot be maintained however that those corpuscles, from which the fatty acids are not recovered, are non-susceptible because of the absence of an intracellular activator, since, as Goebel¹ has shown, these same corpuscles are highly susceptible when suspended in an isotonic sugar solution. These cells do of course contain lecithin. The non-susceptibility of corpuscles in a given instance is not determined by the absolute absence of an intracellular activator, for an activating substance is at all times present (lecithin). The susceptibility or non-susceptibility is determined rather by the *availability* of this substance within the cell as determined by its relation to other constituents of the cell, which relation as shown by Goebel's experiments may vary under differing conditions.

The inhibiting action of calcium chloride for stroma activation, which Noguchi points to as demonstrating that the intracellular activating substance is not lecithin but a fatty acid, is far too variable to serve as an absolute criterion of separation between lecithin and other substances. If it be granted that, in general, the activating action of free lecithin is not inhibited by calcium chloride and that the activating action of the endocomplement-containing stromata is so inhibited, this does not eliminate lecithin as the activating substance in the latter instance. If there is one point which is well established in regard to lecithin it is that this substance behaves in all its reactions very differently in its various protein compounds. The fact that the activating action of free lecithin or even of ovovitellin is not inhibited by calcium chloride affords no basis for a generalization as to what reaction may be displayed by other distinctly different protein-lecithin compounds such as those of the stromata.

In view of the facts: that all corpuscles contain an activating dose of lecithin; that under favorable conditions all corpuscles are dissolved by venom alone; that all such corpuscles contain lecithin, and that this substance is the one substance known to form a lysin with venom, the contention that the intracellular lecithin is the essential endocomplement appears to retain its force.

In contrast to the mass of corroborative evidence concerning the activating action of lecithin are the claims of Bang² and some brief

¹ *Compt. rend. de la Soc. de Biol.*, 1905, 58, p. 420.

² *Biochem. Ztschr.*, 1908, 11, p. 521.

space may best be given to the contradiction and explanation of these claims.

Bang has attempted to show that lecithin itself has no activating action even for the snake venoms. According to this author the apparent activation produced by lecithin preparations is not in fact due to lecithin but to other substances admixed with lecithin and insoluble for the most part in methyl alcohol.[†] The ether-insoluble lecithid described by me, Bang considers a substance pre-existent in commercial lecithin preparations and therefore, according to him, is not formed by a reaction between lecithin and venom.

The materials employed by Bang were of unfortunate selection and to the unsatisfactory character of his so-called lecithin preparations are to be referred the unusual results and deductions which he advances. Throughout that section of his work which purports to be a consideration of the lecithids described by me, Bang has proceeded with a false assumption, namely, that the lecithin which I employed in preparing specimens of cobra lecithid for analysis was crude commercial lecithin such as he himself chose for description and experimentation. As a matter of fact the lecithin which was actually employed in the preparation of the complete cobra lecithids used for chemical analysis was isolated with great care. I refer to those specimens kindly put at my disposal by such authorities on lecithin isolation and structure as P. Bergell and W. Koch, and to specimens isolated by myself. These lecithins contained no substances insoluble either in ether or in alcohol and were, in all instances where the isolation of a pure end product was attempted, several times reprecipitated with acetone immediately prior to their use for the elimination of free fatty acids. When Bang affirms the presence of admixtures in certain crude preparations of commercial lecithin he is discussing facts long since established. When, however, he assumes the presence of such substances in the lecithin actually used by me in preparing lecithids for analysis and deduces therefrom that such lecithids could only be impure products, he is in error.

Further, Bang, in attempting the isolation of lecithin from egg yolk by ether extraction, found that such lecithin as he obtained had

[†] It should be recalled in this connection that certain of the lecithins (cephalin) are insoluble in alcohol (cf. W. Koch).

a relatively slight activating power, and from this concluded that lecithin is presumably not an activator for cobra venom. Here again it appears that Bang was at least unfortunate as to the materials which he isolated and tested, since invariably preparations of lecithin isolated from the same source by fully competent investigators shows a constant activating power for cobra venom (Köch-Bergell). In considering the failure of Bang to isolate an activating lecithin from egg yolk, it is to be noted (1) that Bang relied for his primary extraction entirely upon ether as a solvent, whereas it is well recognized that hot alcohol is the one efficient reagent for the complete extraction of lecithin under such conditions;¹ (2) that his extraction results, where quantitatively controlled, showed wide variations, and (3) that he omits all chemical tests to show that the isolated substances which he assumed to be lecithin were actually such or even in part such.

Had Bang sought by a less tedious and more trustworthy method to determine whether it is the lecithin itself of a given preparation which reacts with cobra venom to form the lecithid, he might have made a direct determination of the lecithin content of the preparation before and after its reaction with cobra venom in the formation of such a lecithid. He would have found that coincidentally with the formation of the ether insoluble lecithid, there is a proportionate disappearance of the lecithin together with the appearance of free fatty acids split therefrom, so that with suitable proportions of venom approximately the entire lecithin content of the preparation is finally exhausted in the formation of the highly lytic end product—lecithid. In elimination of Bang's contentions other proofs of the actual participation of lecithin in a chemical reaction with venom are not wanting and for corroboration of the more obvious instances the reader is referred to the summary given elsewhere.²

Von Dungern and Coca,³ while substantiating the occurrence of a chemical reaction between lecithin and the venom resulting in hemolytic lecithids, have attempted to show that the lecithids them-

¹ Cf. Hoppe-Seyler, *Handbuch*, 7th ed., p. 157; Kyes and Sachs, *op. cit.*; Noguchi, *op. cit.*

² Cf. von Dungern and Coca, *Biochem. Ztschr.*, 1908, 12, p. 407.

³ *Münch. med. Wchnschr.*, 1908, 55, p. 437, and *Biochem. Ztschr.*, 1908, 12, p. 407.

selves are simple split products of lecithin and lack any possible venom constituent which would justify their recognition as toxins.

This claim of von Dungern and Coca is indicated, according to them, by the facts (1) that simple splitting of lecithin in the absence of venom yields hemolytic substances; (2) that the chemical analysis of cobra lecithid approximates that of monostearyl-lecithin, and (3) that the antibody secured in certain experiments performed by them was not an anti-lecithid but an antibody to native venom hemotoxin.

It is indeed surprising, in view of the now extensive literature concerning the hemolytic action of fats and fatty acids, that investigators should attach special significance to the fact that certain products of lecithin, resulting from the splitting of this substance in the absence of venom, should be hemolytic. Just so surely as fatty acid radicals are combined in the lecithin molecule, so surely will any splitting of lecithin freeing such fatty acids allow the isolation of hemolytic substances. But the question in this instance is not, whether lytic substances may be split from lecithin, as indeed they may, but rather whether any such substances possess all the properties displayed by the lecithids. As will be seen later, they do not.

The second point upon which von Dungern and Coca based their conclusions is one to which I drew attention in a previous publication and there discussed.¹ It is true that by present methods of chemical analysis no difference in structure can be detected between monostearyl-lecithin and the lecithids, but it is the universal experience of investigators that direct chemical analyses, as now practiced, are by a long way insufficient for the determination of even the grosser structural characteristics of the true toxins. It may be disappointing that a chemical analysis of the lecithids does not yield explicit data concerning the venom constituent of those compounds, but it is not surprising in view of the fact that chemical methods have not to the present succeeded in revealing the structural peculiarity of a single true toxin to which its specific physiological action may be referred. The molecular weight determination of cobra lecithid shows that the lecithid molecule is one of extreme size, in which many fatty acid radicals are linked together by the hemotoxin constituent, and in the presence of such a preponderance of fatty acid radicals it is not to be

¹ Cf. Kyes, *Biochem. Ztschr.*, 1907, 4, p. 109.

expected that the slight amount of the venom constituent would be susceptible to analysis or even recognition by present direct chemical procedures so notoriously inefficient for the analysis of the true toxins in general.

On the other hand, certain biological reactions, much more sensitive to structural differences in toxins, show clearly that a true toxin constituent exists in the lecithid compound. I refer to the extent and character of the hemotoxic action of the lecithids and to their power of stimulating the production of specific antibodies which effect not only their own neutralization but that of the native hemotoxin from which they are compounded. The elaboration of the latter point requires a comparison of the immunization to cobra lecithid given in section IV with that attempted by von Dungern and Coca, and cited by them as challenging the right of the lecithids to consideration as true toxins.

The comparison shows that whereas by immunization with a complete lecithid I obtained an antibody to that substance, von Dungern and Coca in their experiments failed to obtain such an antibody. The cause of this difference in results is not difficult to determine and is seen to exist chiefly in a difference in the materials used for immunization. The lecithid which I employed was in all instances a fully isolated complete lecithid free from admixtures of incomplete lecithids and native hemotoxin. Von Dungern and Coca on the other hand attempted the preparation of a lecithid by a method which I devised early in the venom work but which I was subsequently forced to modify because of its unreliability in producing a satisfactory yield of complete lecithid (cf. p. 217). Although the modified method was published in detail, von Dungern and Coca chose the older method, and with this method obtained preparations which as shown by their protocols were mixtures of incomplete lecithid, native hemotoxin, and a certain amount of complete lecithid. With such preparations these authors inoculated animals, and after a relatively brief period (twelve days), obtained a serum which, in contrast to that obtained by me, possessed a neutralizing action, not for both native venom and lecithid, but for native venom alone. It was from this finding von Dungern and Coca reached the conclusion that cobra lecithid, as such, does not stimulate the production of a

specific antibody and is not, therefore, a true toxin. Such experiments do not however warrant the generalization which von Dungern and Coca offer.

Considering the fact that the mixtures employed by von Dungern and Coca for immunization contained native hemotoxin, it is, of course, not surprising that these authors should obtain an antibody for this toxin, but such a result furnishes no logical basis for the deduction that a complete lecithid cannot be made to produce an antibody. Such results establish only this, that insufficient immunization with a mixture of incomplete lecithid, native venom, and a certain amount of complete lecithid, may produce at a given point an antibody to the native venom without coincidentally producing an antibody for the complete lecithid. Had von Dungern and Coca prolonged the immunization sufficiently to have obtained antibodies for *all* of the toxins present in their mixtures, they doubtless would have found an amount of anti-lecithid in addition to the anti-native hemotoxin. But performed, as these experiments were, with ill-prepared material and extending in all throughout but twelve days, the results demonstrate only that under sufficiently unfavorable circumstances a lecithid may fail to produce its antibody.

Under more favorable conditions, however, as I have shown in section IV, sufficient immunization with a well-isolated complete lecithid results in the production of a specific anti-lecithid, and this fact in itself establishes the toxin nature of the lecithid. Furthermore, the anti-lecithid so obtained neutralizes also the hemotoxin of the native venom from which the lecithid was prepared, showing that the lecithid embraces a group derived from the native venom hemotoxin.

A phenomenon of especial interest which has been repeatedly observed in venom hemolysis is this, that maximum doses of a given venom may fail to produce hemolysis in instances where a smaller dose of the same venom produces typical and complete hemolysis.

Although the occurrence of this phenomenon is not in dispute, there exists no general concurrence of opinion as to its proper explanation.

Stephens,¹ in 1898, advanced the view that in such instances the

¹ Thesis, University of Cambridge, 1898; also *Jour. of Path. and Bact.*, 1900, 6, p. 273.

maximum doses of venom so modify the cell structurally as to inhibit lysis and the outflow of hemoglobin, and Noguchi¹ more recently has produced experimental data in support of this view. An explanation which I suggested in 1902² on the other hand was based on the quantitative relation between the intracellular lecithin and the venom. It was observed that the phenomenon under discussion occurred chiefly with those species of erythrocytes which contain relatively little available lecithin and also that within a given species the inhibiting action of maximum doses was marked in those individuals where the least intracellular lecithin was available. Furthermore it was shown that where certain susceptible corpuscles which had been treated with maximum doses of venom were removed from the venom and suspended in salt solution, these cells did not undergo hemolysis, but that when complements were added however, hemolysis was pronounced. (Cf. Table 57.)

TABLE 57.

	1 C.C. 5 PER CENT SUSPENSION OF RABBIT ERYTHROCYTES + 1 C.C. 5 PER CENT COBRA VENOM, 2 HRS. AT 37° C., CENTRIFUGALIZED AND WASHED ERYTHROCYTES +			CONTROLS: NORMAL RABBIT ERYTHROCYTES + 0.15 C.C. GUINEA-PIG SERUM OR 0.5 C.C. LAKED GUINEA-PIG ERYTHROCYTES
	a) 0.85 per cent NaCl Solution	b) 0.15 c.c. Guinea-Pig Serum	c) 0.5 c.c. Laked Guinea-Pig Erythrocytes ($\frac{1}{4}$)	
Resulting hemolysis	0	complete	complete	0

The result of the above experiment was taken to mean that after treatment of the corpuscles with the strong venom solution, there was insufficient endocomplement available within the cell to activate the hemotoxin amboceptors taken up by these cells but that upon the addition of a supplementary amount of extracellular complements the activation occurred with resulting hemolysis. In explaining the reduction of the amount of available endocomplement it was suggested that venom constituents which did not unite with the cell to produce hemolysis, but which possessed complementophile groups, appropriated a certain essential amount of the endocomplement.

Now, if in the case of susceptible corpuscles the lack of hemolysis in maximum doses of venom is indeed due to a relation between the

¹ *Jour. Exper. Med.*, 1905, 7, p. 1.

² *Berl. klin. Wchnschr.*, 1902, 39, p. 886.

intracellular lecithin and the excess of venom, the same reaction should appear when the stromata of susceptible corpuscles are employed in a fixed amount for the activation of the venom for non-susceptible corpuscles. In fact this is actually what occurs. Thus a minimum amount of endocomplement which produces lysis of the non-susceptible corpuscles with a moderate dose of venom fails to produce such hemolysis where a maximum amount of venom is employed. But further, if the lack of hemolysis by maximum doses of venom depends upon a relation between the venom and intracellular lecithin, a similar blocking might be expected when extracellular lecithin is employed. In other words, we should expect to find that small doses of extracellular lecithin just sufficient for the activation of moderate doses of venom for non-susceptible corpuscles would fail to produce hemolysis when the amount of venom was increased to the maximum. As a matter of fact, this is the result which obtains.

From these results it might also be expected that the added presence of a certain amount of extracellular lecithin to those corpuscles which contain a relatively small amount of available lecithin would eliminate the blocking of the hemolysis by maximum doses of venom. Such is actually the case. The addition of extracellular lecithin to the suspension of rabbits' corpuscles which are to be tested for a hemolysis blocking eliminates this phenomenon and the corpuscles are dissolved by maximum doses of venom as are those of guinea-pig and man.

The sum total of such results then may be taken as a strong indication that the blocking phenomenon is dependent upon quantitative relationship between the venom doses employed and the available lecithin.

On the other hand, results obtained by Noguchi do point directly to the fact that, in given instances at least, venoms which cannot alone dissolve the corpuscles increase the resistance of cells to certain hemolytic agents. Whether or not the constituents of the venom which effect this modification actually play a rôle in the phenomenon under discussion, is yet to be shown, for Noguchi in his experiments unfortunately did not employ a venom which even in medium doses

caused hemolysis of the corpuscles used. It must not be overlooked that the phenomenon to be analyzed is the failure of maximum doses to hemolyze under conditions where lesser doses do hemolyze.

Sachs¹ has also pointed out the fact that the great variation in amount of the same venom required to effect the inhibition with different individuals of the same species speaks against a simple fixing reaction on the part of the venom. Thus, where the blocking of hemolysis by 0.1 c.c. of a 1 per cent solution of cobra venom occurs with one specimen of rabbit's blood, 10 times this amount is required to produce the same blocking under the same conditions but with corpuscles from another individual. Furthermore a general "fixing" action on the part of venoms cannot be assumed for erythrocytes in general since the great majority of the corpuscles show a susceptibility to maximum as to medium doses of venoms.

Noguchi attaches undue importance, it seems to me, to those experiments in which by heating a venom he removes its blocking action without at the same time destroying its hemolytic action. This result means, according to Noguchi, that the hemotoxin is not the blocking constituent of the venom and further that the substance so destroyed acts only as a fixing reagent. In the first place in multiple repetition of Noguchi's experiments I have been unable to completely remove the blocking power of a venom without producing some diminution of its hemolytic action, and a destruction of hemotoxin, but slightly apparent, might of course well be sufficient to eliminate its deviating action. The exact quantitative results here are difficult to determine. On the other hand assuming that the substance which effects the blocking is other than the hemotoxin, it may well be that its action is that of deviating lecithin. It is by no means established that the active hemotoxin is the only constituent of venom with a lecithinophile group. It might also be considered possible that in heating the venom a certain amount of lecithin is liberated from the proteins of the venom itself, thus increasing the amount of complement. This however, I doubt to be the case.

Altogether, the blocking phenomenon appears as one of extreme complexity and is among those phases of venom hemolysis which require more quantitative experimentation before ultimate conclusions may be drawn.

¹ *Biochem. Centralbl.*, 1906, 5, p. 257.

The true toxin nature of the snake venoms, as indicated by the production of their antitoxins, was established relatively early, and these secretions have from the first played an extensive rôle in experimentation concerning the fundamental problems of immunity. Especially is this true concerning the analysis of the exact nature of the toxin-antitoxin reaction. Because of its adaptability to quantitative experiments *in vitro*, the hemolytic constituent of the venoms has been most extensively employed in this connection.

Myers¹ was the first to study exhaustively the quantitative relations between cobra venom hemotoxin and its antibody. Employing the partial neutralization method of Ehrlich, this investigator obtained results which, like the more recent analogous experiments of Flexner and Noguchi, indicated that this toxin is either highly complex after the nature of diphtheria toxin or that, being simpler, the affinity between it and its antitoxin is slight. The neutralization line as plotted approximated the parabolic curves observed in the fractional neutralization of a weak acid with a weak base (boracic acid and ammonia). It was indeed in this contribution that the first suggestion was made that irregularities in the toxin-antitoxin reaction might be referred to a dissociation of the toxin-antitoxin complex, and much use has been made of these experiments by Arrhenius and his followers who have since elaborated this theme *in extenso*.

The experiments of Myers were performed with care and were well controlled. With the development of new methods and the acquisition of new data concerning the mechanism of venom hemolysis, however, there arose distinct indications for the repetition of such experiments under somewhat modified conditions.

Thus the investigations concerning lecithin as an activator brought to light the fact that an accurate determination of the amount of hemotoxin in a given venom can only be obtained in the presence of an excess of extracellular complement. This condition was not satisfied in the experiments above referred to.

To determine more accurately, then, the fractions of hemotoxin neutralized by successive units of anti-hemotoxin, experiments were performed as follows (Table 58):

A given amount of cobra venom was added to various amounts of Calmette's antivenin and after a time the hemolytic action of each mixture was determined for

¹ *Jour. Path. and Bact.*, 1900, 6, p. 415.

ox corpuscles (non-susceptible) in the presence of a multiple activating dose of lecithin. Such an experiment is the following:

Four mixtures were thus prepared:

I.	1.5 c.c.	1 per cent cobra venom				+ 13.5 c.c. salt solution
II.	1.5	"	"	"	+ 0.75 c.c. antivenin	+ 12.75 " " "
III.	1.5	"	"	"	+ 1.5 " "	+ 12 " " "
IV.	1.5	"	"	"	+ 2.25 " "	+ 11.25 " " "

Each of these mixtures was allowed to stand 15 hours at room temperature, after which various dilutions of each mixture were tested for hemolytic action upon 1 c.c. of a 5 per cent suspension of ox erythrocytes in the presence of 0.2 c.c. of a 0.1 per cent solution of lecithin. The amount of hemolysis produced by each mixture is shown in the following table:

TABLE 58.

Amount from Each Diluted Mixture c.c.	Mixture I Diluted 1/300	Mixture II Diluted 1/200	Mixture III Diluted 1/100	Mixture IV Undiluted
1.0.....	complete	complete	complete	slight
0.97.....	"	"	"	"
0.95.....	"	"	"	"
0.92.....	"	"	"	"
0.9.....	almost complete	almost complete	almost complete	trace
0.87.....	"	"	"	"
0.85.....	"	"	"	"

The computation of the hemolytic doses contained in each of the four mixtures of 15 c.c. gives the following results:

TABLE 59.

Mixtures	Number of Lytic Doses Remaining	Decrease in Number of Lytic Doses
I. (Toxin alone).....	4,800
II. (Toxin + 0.75 c.c. serum)....	3,260	1,630
III. (Toxin + 1.5 c.c. serum)....	1,630	1,630
IV. (Toxin + 2.25 c.c. serum)...	5 (approximately)*	1,625

* Three c.c. of mixture caused complete hemolysis.

From this experiment it appears that each 0.75 c.c. of antivenin neutralized the same amount of venom hemotoxin (1,630 lytic doses). Accurately determined then on the basis of the actual maximum of unneutralized hemotoxin in the respective mixtures, the neutralization of cobra hemotoxin by its antibody corresponds in type to the neutralization of a strong acid with a strong base. In other words, the plot of the partial neutralization displays a straight line and not the parabolic curve obtained with weak alkalis and weak acids. This result, many times paralleled, is of especial interest and its

significance will be discussed at a later point. It shows that the cobra hemotoxin is a simple toxin of marked affinity for its antitoxin.

It has been suggested above, that the results obtained by Myers and by Flexner and Noguchi which do not correspond to those just given were due to a lack of sufficient activating substances in the hemolytic experiments to insure an accurate determination of the amount of unneutralized hemotoxin in the several toxin-antitoxin mixtures. Were this actually the case, a sufficient decrease in the amount of lecithin used in experiments such as the one just given should produce results approximating the findings of these authors. This is indeed the case as shown by the following experiment in which a single activating dose of lecithin was substituted for the excess of lecithin in the above experiment.

TABLE 60.

Mixtures as in Previous Experiment Contain 1.5 c.c. 1 per cent Cobra Venom	Number of Lytic Doses Remaining	Decrease in Number of Lytic Doses
I. (Toxin alone)	2,250	...
II. (Toxin + 0.75 c.c. serum)....	1,380	870
III. (Toxin + 1.5 c.c. serum)....	645	735
IV. (Toxin + 2.25 c.c. serum) ...	0	645

It is to be noticed in the above table that, so far as indicated by the hemolysis, the amount of toxin neutralized by the increasing doses of antitoxin did not appear directly proportionate to the amount of antitoxin added. The results obtained thus are comparable to those of Myers. The error in accepting such results as data concerning toxin neutralization is that the degree of hemolysis displayed by the given mixtures under the conditions imposed, is not a true index of the amount of unneutralized hemotoxin actually contained in such mixtures.

Why a minimum activating dose of lecithin is insufficient to guarantee a full expression of the amount of uncombined hemotoxin present in experiments such as the above, appears to be explained by the power of the toxin-antitoxin complex to deviate a portion of the lecithin, thus leaving an insufficient amount free for the full activation of the unneutralized hemotoxin.

The basis for such an explanation is to be seen in the following experiment:

The minimal lytic dose of each of the following three mixtures of venom and antivenin was determined in the presence of an excess of lecithin.

- | | | | | |
|------|----------|------------------------|-----------|---------------------------------|
| I. | 0.5 c.c. | 1 per cent cobra venom | +4.5 c.c. | salt solution |
| II. | 0.5 " | " " " | +0.5 " | antivenin +4 c.c. salt solution |
| III. | 0.5 " | " " " | +1.0 " | " " +3.5 " salt solution |

Next, $1\frac{1}{2}$ the lytic dose, as thus determined, was added to each of the series of tubes containing 1 c.c. of a 5 per cent suspension of ox corpuscles. With these tubes as indicators the amount of lecithin necessary to effect complete hemolysis was determined in each of three instances. Inasmuch as the amount of unneutralized hemotoxin in all three series was presumably the same ($1\frac{1}{2}$ lytic doses), it might have been expected that the amount of lecithin required for activation would, therefore, be the same. Actually, however, as shown in the following table, a greater amount of lecithin was necessary where the greater amount of toxin-antitoxin combination was present.

Inasmuch as horse serum itself unheated as well as heated activates rather than inhibits venom hemotoxin, cholesterol inhibition by the antitoxin (horse) serum added can be eliminated in the above experiment, and it appears clearly that the hemotoxin-antitoxin complex modifies the hemolytic action of the uncombined hemotoxin by deviating an essential amount of the lecithin hemotoxin complement. In this inhibition of hemolysis by the deviation of the lecithin is to be recognized a process analogous to that which I have previously outlined in explanation of the blocking phenomenon displayed by maximum doses of venom. A similar instance has been observed also by Morgenroth¹ in the case of serum lysins, where the serum amboceptors, neutralized by the anti-amboceptor and present in excess, deviate the complement.

The power of the toxin-antitoxin complex to bind lecithin is also demonstrated in the procedure given below.

It is to be remembered that the venom hemotoxin, when shaken with a chloroform lecithin solution, disappears from its aqueous solution appearing in the chloroform solution from which it is isolated as a lecithid by ether precipitation. Now, when a neutralized mixture of venom hemotoxin and antivenin is shaken with chloroform lecithin, no precipitate appears in the chloroform solution when ether is added. In other words, there is no indication of a transfer of the hemotoxin complex from the aqueous to chloroform solution. It might be assumed, however, that the shaking of such a neutral mixture with chloroform lecithin results in the transfer of a certain

¹ *Centralbl. f. Bakt.*, 1904, 35, p. 501.

amount of lecithin to the aqueous solution to form a lecithin-hemotoxin-antivenin compound. It should then be possible to recover lecithin from such a compound by the alcohol precipitation of the aqueous solution. Such is actually the case, as shown by the following detailed experiment (Table 61):

To 0.5 c.c. of a 1 per cent. cobra venom solution 1 c.c. of an antivenin was added; the whole being diluted to 5 c.c. and allowed to stand at room temperature for 15 hours (solution A). Two c.c. of solution A was then shaken with a chloroform lecithin solution, 1.5 c.c. of the aqueous portion was then recovered, and just the amount of native cobra venom added to make the hemolytic power of this solution (solution B) with an excess of lecithin exactly equal to the hemolytic power of solution A. Solutions A and B were then precipitated with 9 volumes of alcohol and the respective precipitate was redissolved in equal amounts of physiological salt solution. Finally the amount of lecithin necessary for the activation of the lytic dose of these solutions was determined.

TABLE 61.

AMOUNT OF 0.01 PER CENT LECITHIN SOLUTION C.C.	1 C.C. 5 PER CENT SUSPENSION OF ERYTHROCYTES + SINGLE LYTIC DOSE OF:	
	A	B
0.57.....	complete	complete
0.5.....	almost complete	"
0.45.....	marked	"
0.4.....	"	"
0.35.....	medium	"
0.32.....	slight	almost complete
0.32.....	"	marked

The above table shows that solution B, which had previously been treated with chloroform lecithin, lacked the lecithin-deviating power displayed by solution A, which contained the lecithin-free toxin-antitoxin complex.

In the total, the experiments cited give adequate proof that in order to obtain a true estimate of the amount of uncombined hemotoxin in partially neutralized venom hemotoxin mixtures, sufficient extracellular complement must be added to eliminate the complicating factor of complement deviation by the antitoxin-toxin complex; and that when an excess of lecithin is employed to this end, the correct determination of the amount of toxin neutralized by each added amount of antitoxin plots in a straight line and not a parabolic curve.

The general significance of this fact is that in the instance of the venom hemotoxin-antitoxin reaction there is no indication that the reaction is reversible after the manner of the reaction between weak

acids and alkalies and such support as Arrhenius and his followers have drawn from the earlier neutralization experiments with the venoms rests upon what I have shown to be errors in experimentation. The same experiments conducted with the elimination of these errors not only fail to furnish proof for the view which holds that all irregularities appearing in toxin-antitoxin neutralizations are to be explained on the basis of the dissociation of toxin-antitoxin complex, but rather show that in an instance where toxoids and toxons are lacking, the irregularities in neutralization are also lacking and the neutralization follows the law of multiple proportions.

In concluding I wish to construct only a most general summary, recognizing that in this, the one instance where a true toxin is placed tangential to structural chemistry, the major value of the work is most surely the general point of view which it establishes, and in the more or less detached details of experimentation which may find broader application in other investigations. In such a summary the following points may be emphasized:

1. That there is present in all venoms a hemolysin, existing as one of a number of distinct toxins.
2. That this hemotoxin effects hemolysis only in conjunction with a so-called complementing substance which however may be found within the erythrocytes.
3. That so far as at present recognized the activating substances are lecithins.
4. That the reaction between the hemotoxin and lecithin is essentially a chemical reaction resulting in the formation of a complete lysin.
5. That this complete lysin is a true toxin in that it stimulates the production of a specific antitoxin.